THE ROLE OF PITUITARY GROWTH HORMONE IN THE REGULATION OF ALBUMIN SYNTHESIS AND CATABOLISM

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Cape Town by

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University of Cape Town

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INTRODUCTION

Some three years ago I availed myself of an opportunity of pursuing a research project in the Department of Medicine at this Medical School. At that time, a long established interest in the study of plasma albumin metabolism was given fresh impetus by the development of techniques which overcame the problem of measuring rates of albumin synthesis and catabolism under conditions of metabolic change. Interest in the Department had centred mainly upon the nature of the adaptive changes occurring in albumin metabolism in response to altered dietary protein intake, and by the time I was due to take up my appointment many of these adaptive changes had been clearly defined. The factors mediating these changes however remained unknown.

The possibility that growth hormone was, at least in part, responsible for mediating these adaptations was suggested by reports of elevated levels of this hormone in the plasma of children suffering from kwashiorkor. The elevated growth hormone levels were shown to correlate inversely with the degree of hypoalbuminaemia, and the fact that after three weeks of treatment when the albumin concentration had risen to normal levels, the plasma growth hormone was no longer elevated, suggested some form of relationship between albumin and growth hormone. When the literature was consulted, a considerable body of evidence was found which implicated the participation of growth hormone in the regulation of albumin metabolism. All of these studies however had become dated by the development of newer concepts and techniques and a reinvestigation of the role of growth hormone in albumin homeostasis appeared justified. Such a study forms the basis of this thesis, and sets out to answer the following questions:

(i) Does growth hormone deficiency and growth hormone treatment result in any changes in albumin synthesis and catabolism, and if so what is the nature of these changes?

(ii) Are the adaptive changes that occur in albumin metabolism under conditions of protein deprivation and repletion in any way explicable in terms of the actions of this hormone?

To this end, and with techniques valid under non-steady metabolic conditions, albumin synthesis and catabolism was measured in hypophysectomised rats, and in hypophysectomised rats receiving growth hormone replacement therapy.

CHAPTER ONE

HISTORICAL BACKGROUND

It was on the basis of clinical and pathological observations that the pituitary gland was first suspected of participating in the regulation of body growth [1]. Acromegaly, a condition characterised by the abnormal overgrowth of certain parts of the body, first described by Pierre Marie and attributed subsequently by Minkowski to hyperactivity of the pituitary gland, contrasted with a form of dwarfism first described by Paltauf. The deduction that the pathogenesis of these clinical presentations was hypofunction or hyperfunction of the pituitary gland, was made essentially on the basis of the post mortem findings of 'atrophied' pituitary glands, or pituitary tumours. Such observations of course were far from actually demonstrating the presence of a growth promoting factor in the pituitary gland.

More convincing evidence however, was provided in 1909 and later in 1912 by Aschner [2, 3] who, having performed the first successful experimental hypophysectomy, demonstrated that surgical removal of the pituitary gland in young pups prevented their further growth and development. These findings on pups were soon confirmed by Crowe, Cushing and Homans [4] in 1910, and on tadpoles by Allen [5] and Smith [6] in 1916. Studies on the rat were initially less conclusive for some hypophysectomies resulted in obesity, whilst others caused the more familiar post-operative "cachexia". The cause of this obesity, to which attention was first drawn by Aschner, [3] was the subject of considerable debate during the third decade of this century, and was centred mainly around the question as to whether the obesity resulted from the removal of the pituitary gland itself, or from concomitant hypothalamic injury. [7 - 10] As is discussed in chapter 2, the issue was resolved in 1927 by Smith [11, 12] who by devising an alternative operative technique convincingly showed that this post-operative obesity was due to hypothalamic damage alone, and that as in dogs and tadpoles further growth and development was prevented by removing the anterior lobes of the pituitary gland, - findings soon confirmed by other workers. [13 - 15]

Additional evidence for growth controlling properties of the anterior pituitary gland was derived from studies in which simple anterior pituitary extracts were administered to animals in order to obtain exaggerated growth responses. Evans and Long in

1921 [16] were the first / .....

1921 [16] were the first to achieve success in such studies, producing giant rats by daily intraperitoneal injections of an ox pituitary extract. The production of acromegaly in dogs was subsequently reported by Putnam et al [17] and Evans et al [18] in 1929 and 1933 respectively. The rat and canine experiments of Smith, [11, 12] Reichert [19] and Richter [13] between 1927 and 1930 provided the first demonstrations that growth could also be resumed after hypophysectomy if pituitary glands were administered as a replacement therapy. Proof of the existence of an anterior pituitary growth hormone finally came in 1944 with the isolation of the hormone in a highly purified form by Li. [20, 21]

Earlier workers were quick to appreciate that these marked effects on growth and development must herald important consequences for protein metabolism. In 1929 Teel and Watkins [22] observed in the dog that the fasting non protein nitrogen of the blood was lowered after the injection of a pituitary extract. Teel and Cushing [23] showed that such injections also caused a marked diminution of urinary nitrogen excretion and Gaebler [24, 25] confirmed that this reduction in nitrogen excretion was almost entirely accounted for by a fall in urea excretion, a result verified by him some twenty years later with a pure hormone preparation. [26] Experiments with rats provided similar information. Following the injection of simple pituitary extracts a fall in the fasting non-protein nitrogen of the blood, [27] plasma amino acids, [28, 29] and urinary nitrogen [27, 30, 31] was reported, and nitrogen balance studies revealed that pituitary extract administration enhanced the positive nitrogen balance shown by growing rats. [30] All these features were confirmed in 1948 by Li and Evans [32] using a highly purified growth hormone preparation.

Taken collectively these findings were interpreted to mean that under the influence of growth hormone nitrogen was being retained in the tissues in the form of protein, and was confirmed by studies of the chemical composition of body tissues. Lee and Schaffer [30] studied treated and growth hormone treated rats in which the food intake was kept equal in both groups. The treated rats gained more weight and had a larger positive nitrogen balance than the untreated rats. Carcass analysis at the end of the experimental period showed that the nitrogen content of the treated rats on a percentage basis exceeded that of the controls. In a group of treated rats

allowed to eat ad / .....

allowed to eat ad libitum, the features were even more pronounced. Similar findings were also reported by Young [33] and Li and Evans, [32] and contrasted markedly with the situation in hypophysectomised animals in which increased urinary nitrogen excretion, [34] increased blood levels of urea [35] and decreased protein content of the carcass [36, 37] were reported.

By the turn of the half century, as both isotopes and growth hormone [38] became increasingly available for research purposes, more attention was being paid to the dynamic effects of growth hormone on protein metabolism. The past twenty years have witnessed intensive investigation in this field so that any sensible survey of the subject, even were it within the scope of this thesis, would be a formidable undertaking. Thus, having attempted to place the earlier work relating to growth hormone and protein metabolism in some historical perspective, an opportune point is reached where attention can be focussed on the particular protein chosen for study in this thesis, that of plasma albumin.

The first indication that the anterior pituitary gland might play a role in albumin homeostasis was presented in 1938 by Goldberg, [39] who observed that the plasma albumin level was moderately reduced in hypophysectomised dogs. That finding has been confirmed in the dog and in other species in a number of subsequent studies. In a series of investigations in the rat, Levin and co-workers observed that the serum albumin concentration three weeks after hypophysectomy, was 25.5%, [40] 23.4% [41] and 26.1% [42] less than the values obtained in normal rats, whilst a 29% decrease was reported by Leathem [43] after the same time interval. Similar observations were made by Li. After a two week post-operative interval, reductions of 22% [44] and 21.3% [45] from control values were noted. Bernasconi [46] showed that the serum albumin concentration had fallen by 27% of its pre-operative value within five days after hypophysectomy, and that no further significant reduction occurred over the next fifteen days.

Although Pedersen and Carstensen [47] observed only slightly reduced albumin levels in hypophysectomised rabbits and concluded that it was "unlikely that the pituitary played a predominant role in plasma protein regulation", further investigation

did not appear to justify this contention. Thus a 22% fall in albumin concentration was recorded in hypophysectomised dogs by Baker and Miller, [48] and Warner et al [49] observed in one of their dogs that a 27% fall occurred within five days of the operation and that no further fall occurred over the next nine weeks.

These studies, in which albumin levels were observed to fall by approximately 25% from control values within two weeks after hypophysectomy, seemed to offer convincing evidence for implicating the pituitary gland in albumin homeostasis, but it remained to be established that the reduction in concentration had not resulted from some disturbance of the plasma volume. Further investigation provided evidence to suggest that this was in fact not the case.

In the rat, Enerbäck et al [50] demonstrated that the intravascular pool of combined albumin and α_1 globulin had decreased by 24% six to nine days after hypophysectomy, and Ulrich et al [51] showed that the "exchangeable" albumin (i.e. the sum of the intravascular and extravascular albumin pools) in the rat was reduced two weeks after hypophysectomy. In a study of human hypopituitary subjects, Jeejeebhoy et al [52] observed that whereas only one of the four cases investigated had a lowered plasma albumin concentration, three had values for "total exchangeable" albumin that were slightly below the normal range.

It was of course impossible to know from these studies if alterations in albumin synthesis or catabolism were responsible for the hypoalbuminaemia, and, if they were, what their nature was. There remains to this day strikingly little information on this aspect. From a review of the literature only two attempts appear to have been made to define the changes in albumin synthesis or catabolism after hypophysectomy. Deferring a more detailed discussion of these studies to chapter five, it suffices here to merely record the results obtained.

From the behaviour of a tracer dose of ^{35}S labelled albumin in hypophysectomised rats, Ulrich et al [51] concluded that after hypophysectomy a reduction in albumin synthesis occurred initially, followed by a reduction in catabolism. In the study on hypopituitary subjects referred to above [52] ^{131}I labelled albumin was used as the

tracer and in four / .....

tracer and in four subjects three had rates of albumin catabolism and "synthesis plus transfer" that were either at, or slightly below, the lower limit of normal.

Hypophysectomy producing as it did a multihormonal deficiency state, it remained to be ascertained which hormonal deficiency or deficiencies were responsible for the disturbances in albumin metabolism seen after hypophysectomy. The belief that growth hormone might at least be partially responsible for these observations was justified on the basis of the evidence cited earlier in this chapter in relation to its effects on overall protein metabolism, and led to studies of the effect of growth hormone treatment on albumin metabolism in particular, both in the intact state and as a replacement therapy after hypophysectomy.

In the intact dog Campbell et al [53] found that administration of growth hormone for seven days produced a fall in the plasma albumin concentration but an increase in the intravascular pool size. In the intact rat Hoch-Ligeti and Irvine [54] observed no change in albumin concentration after a single large dose of growth hormone, and only small increases were observed by Bernasconi [55] after a ten to twenty day period of treatment.

The first study in which growth hormone was used as a replacement therapy after hypophysectomy was that of Li and Reinhardt [45] in which the daily administration of the purified hormone over two weeks was shown to increase the albumin concentration to a level approximately halfway between the normal control value and the value obtained in untreated hypophysectomised rats. Warner et al [49] however were unable to show any effect of growth hormone on the hypoalbuminaemia of hypophysectomised dogs after two weeks of treatment. Enerbäck [50] demonstrated in hypophysectomised rats that whilst nine days of hormone treatment had no effect on the low albumin concentration, the intravascular pool of albumin remained within the normal range.

Studies designed to define the action of growth hormone in relation to albumin metabolism have led to conflicting results, and again it suffices merely to record these varying results, deferring a more detailed discussion to a later chapter.

Firstly in intact/ .....

Firstly in intact animals, Bartlett and Gaebler [58] concluded that growth hormone stimulated albumin synthesis since more ^{15}N glycine was incorporated into albumin in dogs treated with growth hormone than in untreated controls. Using ^{131}I labelled albumin, Gross et al [56] observed no effects of growth hormone treatment on albumin synthesis or catabolism, and concluded that the increase in plasma albumin concentration that was demonstrated was due to reduced capillary permeability which restricted the transfer of albumin from the intravascular to the extravascular compartments. In a clinical study of four adult males, Gabuzda et al [57] showed that growth hormone administration reduced albumin catabolism but had no effect on albumin synthesis.

Equally varying results were obtained in studies where growth hormone was replaced after hypophysectomy, for in rats growth hormone was shown to stimulate albumin synthesis, [51] but rates of catabolism and "synthesis plus transfer" already low in hypophysectomised humans, were further lowered on growth hormone treatment. [52] A final study that requires to be mentioned is that of Sellers et al [59] who measured albumin synthesis in the isolated perfused rat liver by the ^{14}C carbonate method, and found a 33% increase in synthesis rate if the donor animal was pretreated with growth hormone.

We have thus seen that what little information is available on the effects of growth hormone deficiency and treatment on albumin metabolism is conflicting in nature. To a great extent this reflects the shortcomings of the methods used in the investigations, as will be discussed later. Relatively recent technical advances now permit more meaningful investigations of albumin metabolism, and a reinvestigation of the effects of hypophysectomy and growth hormone replacement therapy with these newer techniques forms the basis of this thesis.

CHAPTER TWO

A GENERAL ACCOUNT OF THE PRINCIPAL METHODS EMPLOYED IN THIS THESIS

1. HYPOPHYSECTOMY:

In the rat hypophysectomy is most easily performed via the parapharyngeal approach. The transbuccal approach described by Aschner in 1909 [2] and 1912 [3] is suitable for use in dogs, but for anatomical reasons is not feasible in the rat. A method employing a temporal approach was highly favoured in the third decade of this century and was used on dogs and rats but did not prove to be entirely satisfactory. As used in the rat, the temporal lobe of the brain is lifted to expose the carotid artery, occulomotor nerve, hypothalamus and pituitary stalk. A fine hypodermic needle is inserted through the diaphragma sellae, and 0.01 ml. of a 5% solution of chromic acid injected into the gland. This method gave inconstant results due to incomplete ablation or to hypothalamic damage, the latter, as was mentioned in chapter one, often resulting in gross obesity. Its high mortality and morbidity and poor results, led Smith to devise the parapharyngeal technique which is described in detail in his monumental work of 1930. [12] This method is highly satisfactory and although it has been modified slightly, in essence forms the basis of the method universally employed today. This modified method is described by Ingle et al, [60] and has provided, with all but a few further modifications, the basis of the technique employed in this thesis.

The method of transauricular hypophysectomy first described in 1931 by Koyama [61] has received scant attention. This method provides for the aspiration of the gland by means of a hypodermic needle and syringe passed through the wall of the external auditory meatus to lie in the pituitary fossa. Major disadvantages of this technique are incomplete aspiration, especially in large rats, damage to the blood vessels at the base of the brain and secondary bacterial infection. In order to overcome these disadvantages Falconi and Rossi [62] have designed special needles sized according to the weights of the experimental animals, while Gay [63] has adapted the ear bars of standard stereotaxic frames to act as a guide to the needle, the proper angle of entry thereby being determined not by the skill of the operator, but by

the design of the / .....

the design of the apparatus. Neither modifications however seem likely to displace the parapharyngeal method as the method of choice. A detailed illustrated account of the parapharyngeal technique of hypophysectomy as employed in this thesis, is contained in the appendix.

II. IODINATION:

For the valid use of tracer molecules in metabolic studies, the fundamental requirement that needs to be satisfied is that the introduction of the label into the molecule must be achieved in such a fashion that the body fails to distinguish metabolically between the tracer molecules and its own unlabelled molecules. Intensive research has led to a greater standardization of the conditions under which albumin may be labelled reliably. Damage to labelled albumin molecules may occur during any of the successive stages in their preparation, and it seems a convenient approach to consider these in turn.

(a) Extraction of albumin from Plasma

The susceptibility of albumin to damage from pH values of less than 4 or greater than 10, and to contact with organic solvents [64, 65] places some restrictions on the choice of fractionating methods. Whilst Korner and Debro [66] believe that the albumin obtained from their extraction process with TCA-ethanol is native albumin, the low pH (pH 1-2) to which the albumin is exposed, seems to preclude the use of this method. For this reason the same would seem to apply to the HCL-ethanol method, [67] though the experience of Sutherland [68] indicates that albumin prepared by this method is not denatured. Salt precipitation, as for example with ammonium sulphate, [69] no matter how often repeated, appears to be a safe method of fractionation. Despite the fact that a percentage of globulins contaminate the albumin fraction thus obtained, after labelling these contaminants can be reduced to insignificant levels by adding unlabelled plasma and repeating the precipitation procedure. [64]

(b) Iodination

Detailed reviews of the chemistry of iodination have been presented by Hughes [70, 71] and Helmkamp et al. [72, 73] Only cationic iodine is available for substitution into the benzene ring of tyrosine. This may be formed from iodide with oxidising

agents such as / .....

agents such as hydrogen peroxide or chloramine T. Whilst the chloramine T method [74] has been used with success for the labelling of polypeptide hormones, oxidising agents appear to be injurious to plasma protein molecules and should therefore be avoided. [65]

Suitable alternative methods rely on the formation of hypoidous acid (HOI) in aqueous solution as an essential prerequisite for labelling. Iodide-iodate methods [65, 75] which yield HOI have a theoretical efficiency of labelling of 50%, but if iodine monochloride [76] is used to yield HOI, the efficiency of labelling is theoretically 100%. The stability of the iodine monochloride solution, as well as the simplicity of the method as a whole, has led to its widespread use.

At alkaline pH iodine monochloride in aqueous solution yields HOI. Exchange between radioiodine and HOI yields HO^{131}I, and protein labelling proceeds with rapidity once this solution is introduced into the protein solution. Rapid mixing of these two solutions is necessary to obtain uniform labelling, but in the light of experience that has shown increased efficiency of iodination, [64] and reduced possibilities for protein denaturation [65] if the volumes of the reactants is kept small, the elaborate counter-current jetting apparatus devised by McFarlane [65] has been dispensed with. The iodine monochloride method has been scrutinised by Helmkamp et al [72, 73] and the conditions for maximum efficiency of labelling have been clearly defined.

(c) Removal of radioiodide

Whilst the theoretical efficiency of the iodine monochloride method is 100%, in practice efficiencies of 60 - 70% are achieved. Residual free radioiodide may be effectively removed by passage through a chloride ion exchange resin. Though the possibility of some surface denaturation cannot be excluded, [65] the preliminary addition of plasma to the column will minimise this risk. When however proteins are being labelled for turnover studies, for which standards of protein preparation may not be compromised, removal of radioiodide is best performed by dialysis against distilled water.

(d) Self irradiation / .....

(d) Self irradiation damage

Albumin which has been subjected to radiation has evidenced abnormal chromatographic [77, 78] and metabolic [79] behaviour. Whilst adequate protection from self irradiation damage may be achieved by the addition of unlabelled carrier protein, [77] as a rule preparations should be put to use as soon as possible after labelling.

(e) Sterilisation

Heat sterilisation of albumin is not permissible. [80] Seitz filtration and other filters operated by suction have been widely used, but suffer from the disadvantages that much of the sample may be retained in the filter bed despite washing through, and that excessive frothing may be deleterious to the protein preparation. Both of these objections are easily overcome by the use of "millipore" filter units.

Proof of the satisfactory biological nature of the labelled preparation implies a comparison with the unlabelled protein, which as Hughes has remarked, [71] is "obviously a circuitous proposition". It is clear that grossly misleading results could be obtained from the use of unsatisfactory albumin preparations. Whilst damage to grossly overiodinated albumin molecules may be detected by changes in absorption spectra and water solubility, [70] and in electrophoretic mobility, [81] molecules suffering lesser degrees of damage whether due to lesser degrees of substitution (a mean of 1 atom iodine per molecule is the accepted safe level of substitution for albumin), or to any of the other possibilities outlined above, may show no abnormal physiochemical properties but behave abnormally "in vivo". [77, 82 - 84]

"In vivo" the most reliable test of iodine labelled albumin consists of a comparison of its plasma half life with that of biosynthetically labelled ^{14}C albumin. Rapid respiratory and renal excretion of ^{14}C reduces reutilisation of this label to a negligible extent, and good agreement between the "in vivo" behaviour of iodine and ^{14}C labelled albumin has been obtained in rats [85] and rabbits. [65, 86, 87] This approach has not only made it possible to define the normal range of turnover

values for these / .....

values for these species, but has also secured a place for iodine as a valid tracer substance, provided the various precautions mentioned above are taken when preparing the labelled molecule.

Comparison with a ^{14}C labelled albumin preparation as a routine check on iodine labelled preparations would hardly seem justified in view of the time and expense involved. Since damage to albumin molecules always results in more rapid but never slower rates of elimination [88, 89] an abnormally high percentage of radioiodide in the urine, or retained in the plasma on the first day or two of the experiment, would provide alternative evidence of protein damage. With the proviso that breakdown products are not retained in the body water, in cases of severe damage a short lived rapid component may also be detected in the total body activity graph. It must be emphasised that minimal damage may manifest as nothing more than slightly increased rates of catabolism.

As will be evident from the description contained in Chapter 3 and in the appendix, the methods employed in this thesis were based on the principles outlined above, all possible precautions being taken to ensure a satisfactory labelled albumin preparation. The final test remained as its behaviour "in vivo". Albumin turnover and plasma half lives were measured in a large group of normal rats, and the values thus obtained agreed well with those observed by other workers (Chapter 4). When turnover measurements were to be made under experimental conditions, the experiments were designed so that turnover was first measured during a normal pre-operative control period. It was always established that these pre-operative turnover rates fell into the normal range before embarking on the experimental procedures. In this way it was possible to be certain that any subsequent slowing of catabolism that might be observed during the experimental period was not due to the completed excretion of more rapidly eliminated denatured molecules.

III MEASUREMENT OF ALBUMIN CATABOLISM

Many earlier estimates of albumin turnover were made with biosynthetically produced tracers. Biosynthetic labelling was achieved by the intravenous administration of ^{14}C / .....

stration of ^{14}C or ^{35}S labelled amino acids which were then utilised by the liver for albumin synthesis. Since the incorporation of the label into the albumin molecule occurred "in vivo" there could be little doubt that the label was native. Despite this, values for albumin turnover and plasma half lives varied considerably. These discrepancies were correctly attributed to reutilisation of the label.

After the administration of a labelled amino acid, plasma proteins and proteins of all body tissues become labelled. As turnover continues and these tissues are broken down, the label re-enters the free amino acid pool and once again becomes available for albumin synthesis, thereby prolonging the observed plasma half life. Pen et al [90] showed convincingly that the degree of reutilisation was greatly influenced by the choice of labelled amino acid. Essential amino acids being more efficiently conserved displayed a high degree of reutilisation and hence longer plasma half lives than non essential amino acids.

Reutilisation could be greatly reduced, and in the case of ^{14}C proteins to negligible levels, if after the administration of the labelled amino acid, the albumin thus produced was isolated and transferred to another animal. This procedure first employed by Hevesy and Hahn in their work on phospholipids, is described in full by Goldsworthy and Volwiler. [91] Unfortunately since only a small percentage of the injected amino acid becomes incorporated into albumin the technique is wasteful and becomes expensive if a sufficient yield is to be obtained. To do this the labelled amino acid must be administered in large doses, which whilst permissible for experimental animals, is precluded in humans because of the radiation risk involved.

The use of iodine as a tracer label has much to commend it. In the first instance, provided that the thyroid gland is "blocked" by the administration of inactive iodide, reutilisation of the label does not occur. [86, 82] Secondly, the label is rapidly and quantitatively excreted in the urine, providing the means whereby catabolism may be measured by a relatively simple method based on urinary excretion data not possible with biosynthetically produced tracers. Additional advantages reside in the relative simplicity of the techniques with which iodine may be tagged to albumin "in vitro", and the ease with which biological samples may be assayed for radioactivity.

It is relevant at this / .....

It is relevant at this point, before describing the methods by which albumin catabolism is measured, to consider briefly the fate of a tracer dose of albumin after its introduction into the blood stream. Immediately after intravenous injection, the dose rapidly becomes spread throughout the intravascular space. It may be assumed that within the first few minutes after injection, no significant loss of labelled molecules occurs into the extravascular spaces, so that the extent to which the dose is diluted will provide a measure of the size of the plasma volume. Almost immediately thereafter plasma radioactivities will be observed to diminish. If these radioactivities are plotted on semilogarithmic paper, the initial portion of the graph will be observed to consist of a curve which after a variable time resolves itself into a straight line, Fig. 2:1. The fall in the initial curved portion of the graph * results from the simultaneous onset of two processes:

(i) loss of labelled molecules into the extravascular spaces and

(ii) catabolism.

The final linear component reflects catabolism alone, but whilst the slope of this component provides an index of catabolism, for reasons to be discussed below, this slope alone is insufficient for actual quantitation of the catabolic rate.

Transfer of labelled molecules from intravascular to extravascular compartments results in albumin being widely distributed throughout the body (in lymph channels, serous cavities, cerebrospinal fluid etc.) from which return occurs via the main lymph channels in the thorax, so that under steady conditions the amount of albumin passing from intravascular to extravascular compartments is balanced by an equal amount returning in the opposite direction. Despite its widespread distribution, it has been established that albumin is only catabolised in relation to the intravascular compartment. Evidence suggesting intravascular catabolism was first provided by Berson and Yalow [92] who observed that the daily urinary excretion of the label was a constant fraction of the activity remaining in the plasma over that same period, but bore no relation to the concentration of the label in the extra-

vascular compartment / .....

* The curved portion of the graph, occurs when there is a net flow of labelled molecules from the intravascular to extravascular compartments. This lasts for a variable period of time - usually not less than 48 hours, and is frequently referred to as the Distribution Period.

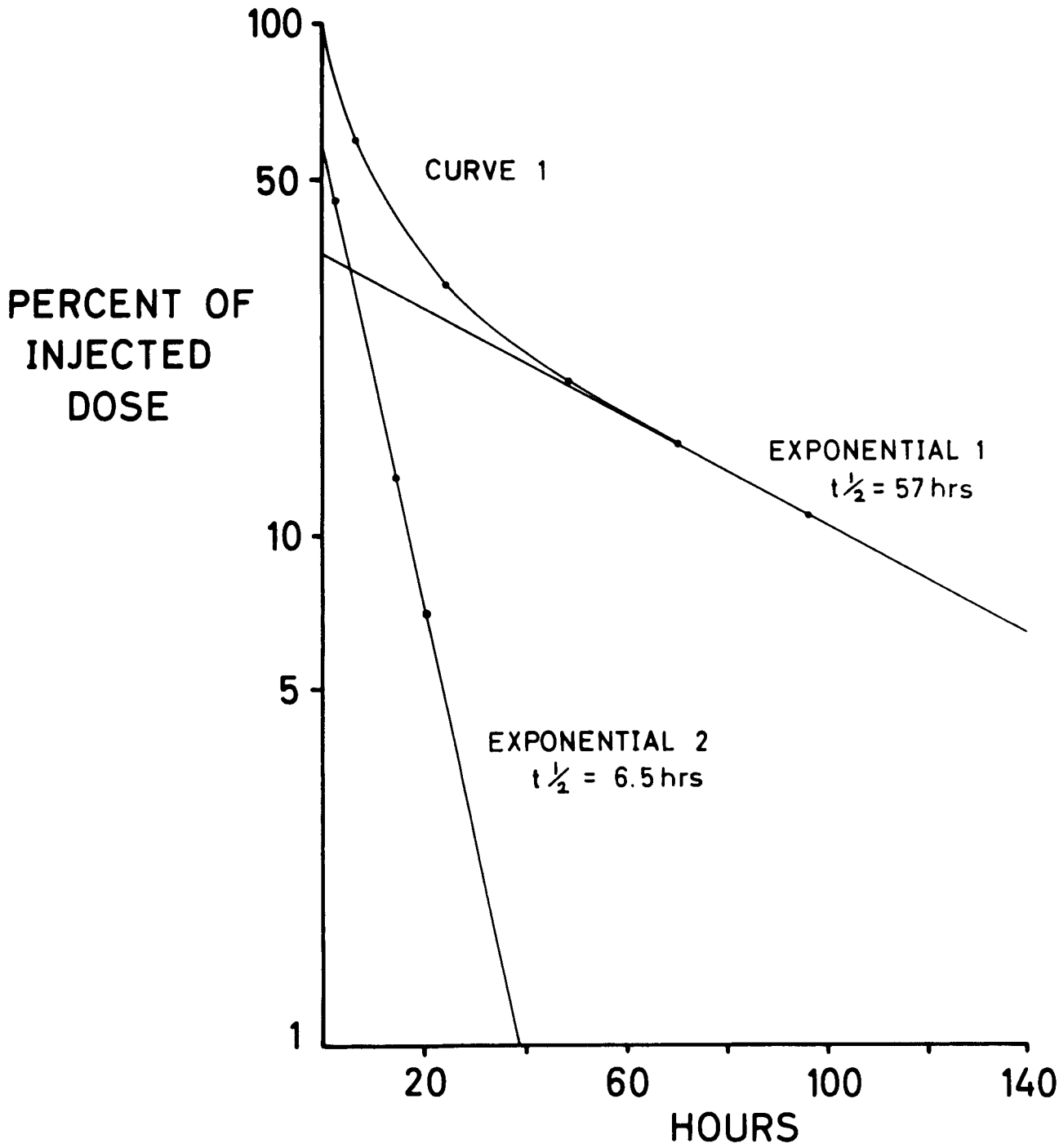

Figure 2:1 Graphic resolution of a plasma albumin specific activity curve into its two exponentials.

vascular compartment or whole body. Campbell et al [85] and McFarlane [89, 93] confirmed this observation and it became accepted that catabolism occurred intravascularly, or at least in tissues in close metabolic proximity. In such a situation it will be clear that specific activity differences must exist between the intravascular and extravascular compartments. There will always be a net transfer of labelled molecules from the extravascular to intravascular compartments where they are catabolised. Since albumin of higher specific activity continually enters the intravascular compartment from the extravascular compartment, the plasma half life will be prolonged and will underestimate the true rate of decline of intravascular activities, and the metabolic rate.

Methods for the calculation of the albumin catabolic rate fall broadly into two main categories. The first category contains a group of methods which are based on the analysis of plasma radioactivities alone. The second category makes use of both urinary and plasma radioactivities for the purpose of calculation.

Category 1: The first measurement of albumin catabolism in man was carried out by Sterling [94] and was based on the behaviour of the terminal exponential part of the plasma radioactivity curve. Data thus obtained would only acquire validity in the event of catabolism occurring at equal rates in both intravascular and extracompartments, since this method assumes that specific activities in both compartments are equal at all times. From the evidence cited above, such a situation clearly does not obtain.

In its simplest form, the model describing the synthesis, catabolsim and distribution of albumin is a two compartment open mamillary system, (Fig. 2:2). The intravascular compartment to which albumin is added by liver synthesis and from which losses due to catabolism occur, exchanges reversibly with a single extravascular compartment. The mathematical equations describing this situation were first presented by Rescigno, [95] and the information required for their solution was obtained by graphic analysis of the plasma radioactivity curve. [75] In most instances the plasma curve resolved into two first order components and was described by the equation:-

$x = / \ldots\ldots\ldots\ldots\ldots\ldots$

$$x = c_1 e^{-b_1 t} + c_2 e^{-b_2 t}$$

where x = isotope concentration in plasma at time t

where c_1 and c_2 = the y axis intercepts of the two components

where b_1 and b_2 = the slopes of the two components

from which the following expression for the calculation of the fractional catabolic rate was derived:-

$$\frac{b_1 \cdot b_2}{c_1 \cdot b_2 + c_2 \cdot b_1}$$

It can be seen from this expression that information from both components of the graph is required for the calculation of the fractional catabolic rate and serves to emphasise the inappropriateness of the Sterling model based on the terminal exporential alone. As increasingly complex kinetic models have been proposed [75, 96 - 99] plasma radioactivity curves have been subjected to more and more sophisticated forms of analysis often requiring the aid of analogue computers. McFarlane [64] has referred to some of the difficulties of this form of analysis, which in addition, in non steady states becomes exceedingly complex. [100]

Category 2: The use of urinary radioiodide excretion data makes it possible to calculate the catabolic rate under both steady and non steady states with relative ease. As albumin molecules are catabolised, the released iodide accumulates in the body water until the renal iodide threshold is reached, and since the urinary route is for practical purposes the sole route of iodide excretion, the amount appearing in the urine will provide a quantitative estimate of the amount of albumin catabolised. Although iodide (the principal form in which the label is liberated after catabolism) is concentrated and secreted in salivary gland and gastro-intestinal secretions, it is extensively resorbed in the gut and the amount of iodide excreted in the faeces may be neglected under normal circumstances. This method (frequently referred to as the Clearance Method), was first used by Berson and Yalow [82, 92] and has the advantage that the catabolic rate may be calculated on a daily basis, thus enabling shortlived fluctuations to be detected.

The daily fractional / .....

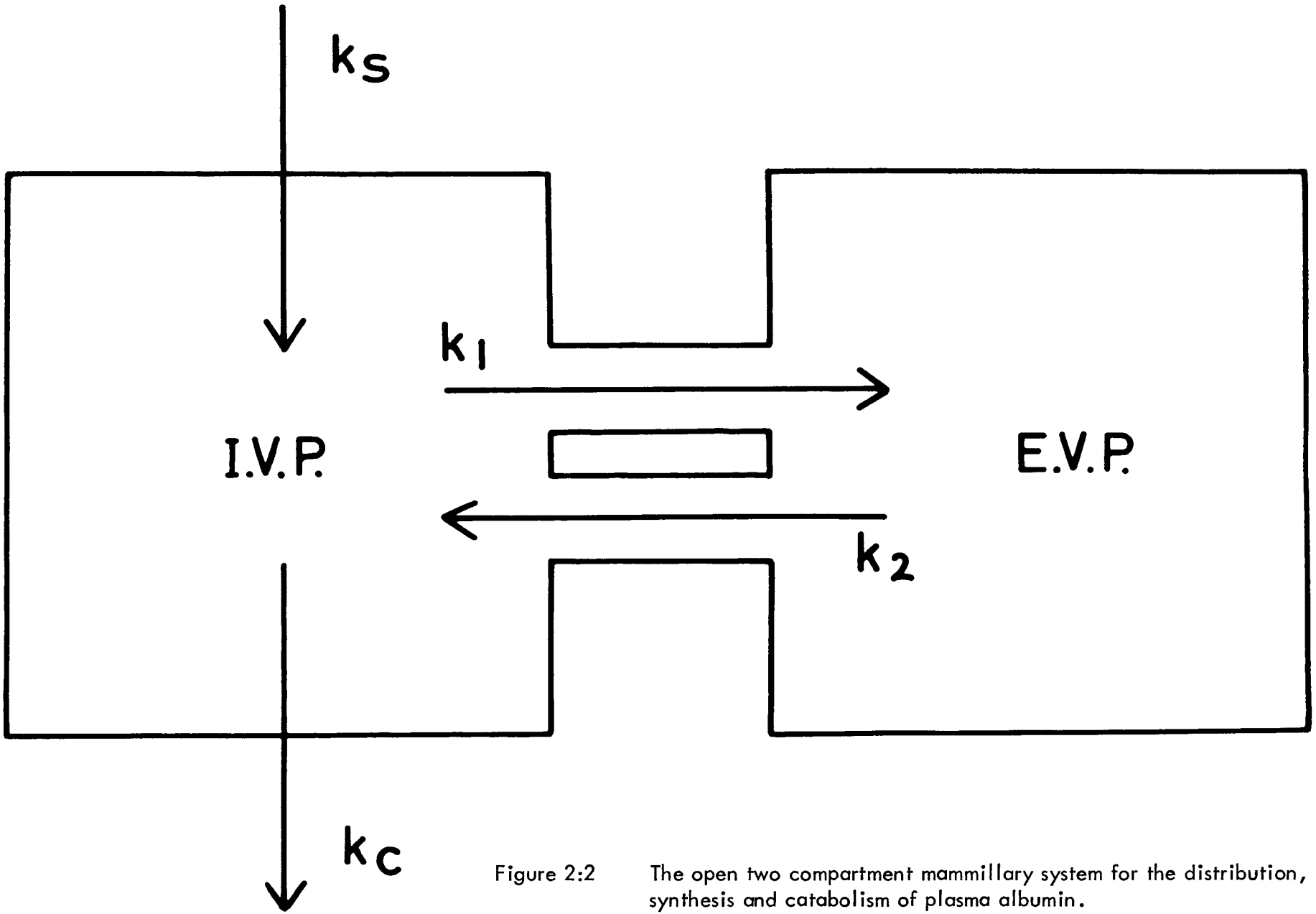

Figure 2:2 The open two compartment mammillary system for the distribution, synthesis and catabolism of plasma albumin.
I.V.P. = Intravascular pool, E.V.P. = Extravascular pool.
k_s = rate constant for albumin synthesis, k_c = rate constant for albumin catabolism, k_1 and k_2 = rate constants for transfer from I.V.P. to E.V.P., and E.V.P. to I.V.P. respectively.

The daily fractional catabolic rate (as percentage of the intravascular pool) is that fraction of the protein bound activity in the plasma that is excreted daily in the urine. Knowing the size of the intravascular pool of albumin, an estimate of the absolute amount of albumin catabolised can be obtained. (89)

The absolute catabolic rate may also be calculated under non steady conditions in which the intravascular pool is changing, by expressing the plasma data as albumin specific activities. This method is valid under non steady states since the only assumption made is that the specific activity of albumin in the plasma is the same as that at the sites of catabolism. (101)

Extravascular albumin

The presence of a large mass of extravascular albumin has implications for both biophysicist and clinician alike. Its subdivisions and the rates of exchange with the intravascular compartment are of importance to the kineticist interested in providing the most representative model of albumin metabolism, whilst estimation of its size is of importance to the clinician since in disease states when abnormal losses of albumin occur from the intravascular compartment, albumin is transferred from the extravascular compartment to the intravascular compartment in an attempt to mitigate these losses. Methods of measuring the extravascular pool of albumin have been reviewed by Schultze and Heremans. (102)

MEASUREMENT OF ALBUMIN SYNTHESIS

The direct measurement of the rate at which a protein is synthesised can be achieved by measuring the rate at which a labelled precursor amino acid is incorporated into that protein. Since iodine is never used for protein synthesis, amino acids bearing this label can never provide direct information about rates of protein synthesis. Albumin synthesis rates have frequently been inferred from the "in vivo" behaviour of tracer doses of iodinated albumin. Under steady state conditions it is permissible to infer the synthesis rate after a measurement of the catabolic rate, since obviously under these conditions the two processes proceed at the same rate.

Under non / .....

Under non steady state conditions however this simple expedient is no longer possible, and various methods of analysis have been proposed in attempts to determine the separate contribution of synthetic and catabolic processes to observed radioactivities.

The major obstacle to the direct measurement of plasma protein synthesis rates, is the need for accurate measurement of precursor amino acid specific activities. Since these specific activities are those at the intracellular site of protein synthesis, and not of the amino acid as it circulates in the blood stream, it is necessary to obtain tissue samples for measurement. In the case of albumin, specific activities would have to be made on biopsy samples of liver tissue. Furthermore since after the administration of a single dose of labelled amino acid, precursor specific activities may display considerable fluctuation, repeated biopsy samples would be necessary in order to define the precursor specific activity curve. In the face of these difficulties many investigators have been obliged to rely on various indirect methods of measuring albumin synthesis, to which attention will be drawn at the end of this chapter.

Yet it is possible to overcome this seemingly insoluble problem of sampling by the use of external indicators of intracellular precursor specific activities. This approach based on the precursor-product relationship will be discussed further in a later paragraph. In respect of albumin production, urinary hippuric acid has been proposed as an external indicator of intrahepatic glycine specific activity [103] and urea [104] has been proposed as an external indicator of intracellular arginine guanidine carbon specific activity. Recently the technique for measuring albumin synthesis by comparing the rates of incorporation of cystine and cysteine into albumin with the rates that these two amino acids are oxidised into inorganic sulphate has been revived, [134] and arterial total $^{14}CO_2$ after ^{14}C carbonate infusion has recently been suggested as an alternative to urea as an external indicator of intracellular arginine specific activity. [135]

The proposal to use urea as an external indicator of intracellular specific activity arose out of the earlier work of Delluva and Wilson [105] who demonstrated that after its administration, $^{14}CO_2$ condensed with ammonia to give by way of

intermediates in the / .....

intermediates in the Krebs-Henseleit cycle, arginine that is labelled only at the guanidine carbon position (Fig. 2:3). Arginine may then be used for albumin synthesis [104] or the guanidine carbon may be split off by the action of arginase to provide the single urea carbon atom. Since the production of urea from arginine is many times greater than the protein produced from this source, and since urea rapidly leaves the hepatic cells, urea may be regarded as a reliable external indicator of intracellular arginine guanidine carbon specific activities. [64]

In 1963 McFarlane [69] and Reeve et al [106] separately reported a method for the direct measurement of albumin synthesis (and other proteins produced solely by the liver) which by exploiting this metabolic relationship between the guanidine carbon of free intracellular hepatic arginine and albumin arginine, and of urea carbon, obviated the need for measuring intracellular specific activities.

In general terms, if P is the mean precursor specific activity (μc/g) throughout a given time interval during which a product containing M grams of precursor and R μc is produced, then R = MP and rearranging $P = \frac{R}{M}$. Furthermore this relationship holds good (vide infra) for all products of the same precursor, so that

$$P = \frac{R}{M} = \frac{R_1}{M_1} = \frac{R_2}{M_2} = \ldots\ldots\ldots\ldots \frac{R_n}{M_n}$$

More specifically for the case of albumin, since albumin arginine guanidine carbon and urea carbon are the two products of a single precursor namely, intracellular arginine guanidine carbon, then

$$\frac{R \text{ albumin}}{M \text{ albumin}} = \frac{R \text{ urea}}{M \text{ urea}}, \text{ at a given time (t)} \quad \text{(1)}$$

where M albumin = mass of albumin guanidine carbon synthesised

M urea = mass of urea carbon synthesised

R albumin = total activity in albumin guanidine carbon

R urea = total activity in urea carbon

The validity of this precursor product relationship, which states that the ratio of mass to radioactivity in products of the same precursor is constant, has been demonstrated in mathematical terms by Reeve et al [106] and Reeve. [107]

Since the total / .....

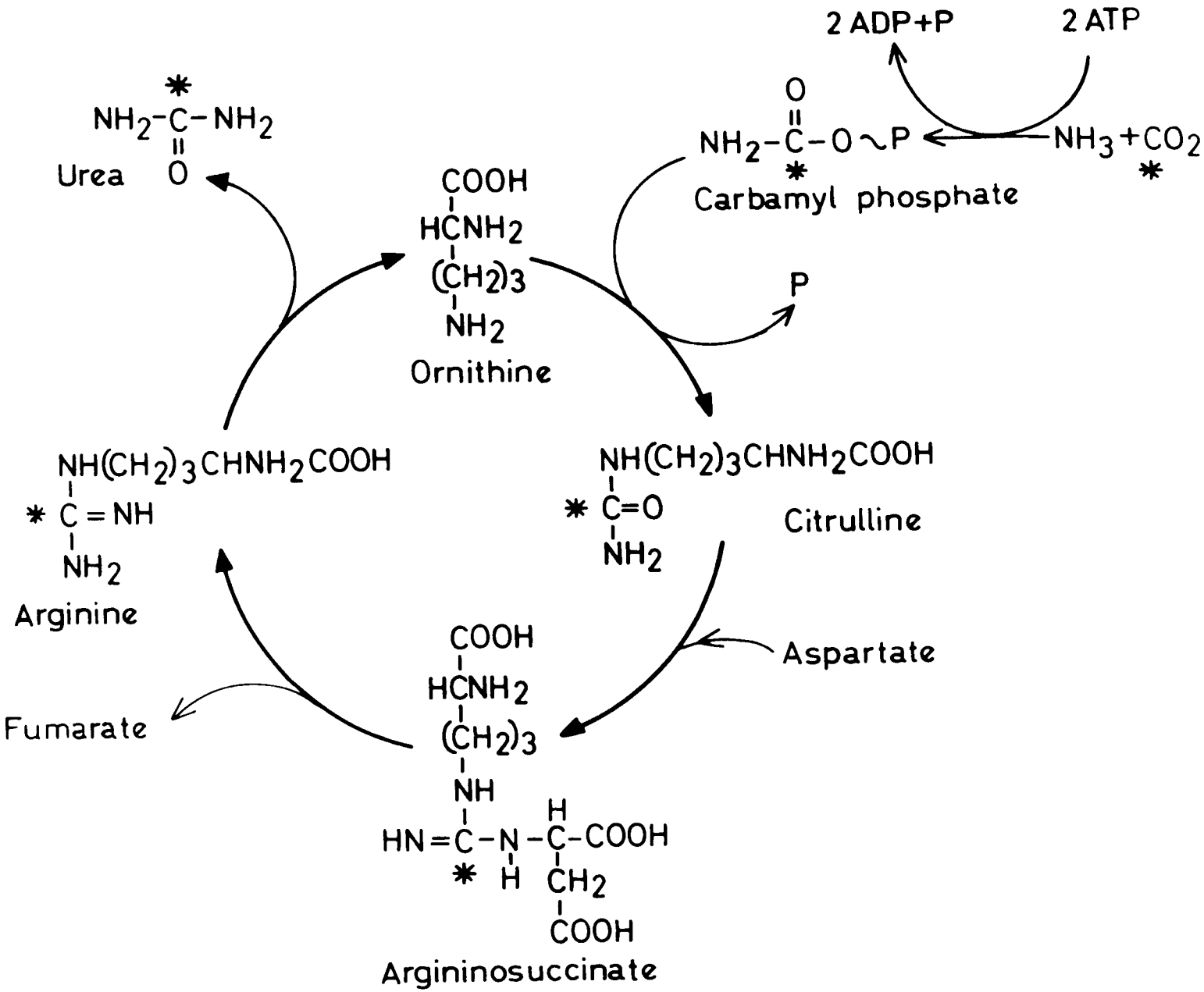

Figure 2:3 The Krebs-Henseleit Cycle for urea metabolism. The pathway of the carbon atom entering as CO_2 is marked by an asterisk.

Since the total activity appearing in each of these two products in a given time will be proportional to their absolute (mass) rates of formation, it is possible to deduce the absolute rate of albumin synthesis if the total activity incorporated into the two products, and the absolute rate of urea synthesis are known. Difficulties have however been encountered in the accurate measurement of the absolute rate of urea synthesis. In view of endogenous catabolism of urea by bacterial action in the gut, the amount of urea excreted is considerably less than that synthesised [108, 109] and it is not possible as has been suggested [69] to infer the absolute rate of urea synthesis from the mass of urea excreted. Koj et al [110] suggested that the mass rate of urea synthesis could be obtained from the product of the fractional rate of urea synthesis and the body urea pool. As was shown by Regoeczi et al [109] uncertainty exists about the accuracy of measurements of the size of the body urea pool by any of the available methods, since estimates based on a ^{14}C urea dilution technique differed from those based on body water measurements. The latter method in addition assumes that urea is present throughout the body water at the same concentration as in the plasma, which in that study at least was not shown to be the case.

In the light of these difficulties a modified formula has been proposed by McFarlane [111] in which total activities are substituted for by specific activities (S.A.) and which provides for the determination of fractional rates instead of mass rates of synthesis. In this way the need for measuring the body urea pool is dispensed with.

Rearranging equation (1)

$$\frac{\text{M alb.}}{\text{M urea}} = \frac{\text{R alb. at t}}{\text{R urea at t}}$$

By arbitrarily dividing both sides of the equation by the sizes of the urea carbon and guanidine carbon pools, the following working formula is derived:

$$\frac{\text{FSR alb.}}{\text{FSR urea}} = \frac{\text{SA alb. at t}}{\text{SA urea at t}}$$

and by rearranging

$$\text{FSR alb} = \frac{\text{FSR urea} \times \text{SA alb. at t}}{\text{SA urea at t}} \qquad (2)$$

where FSR = fractional synthesis rate

This relationship / .....

This relationship will only be true if the system is closed. Since however losses of both urea and albumin occur from the intravascular compartment, observed specific activities at time 't' have to be corrected to obtain values that would have existed had no such losses occurred. The assumptions and workings of the method by which albumin is measured directly will now be discussed in general terms.

^{14}C carbon guanidine arginine may be introduced into the liver by either administering the labelled amino acid as such, or by administering ^{14}C sodium carbonate from which $^{14}CO_2$ is liberated and incorporated into arginine via the urea cycle. These two approaches have been evaluated by McFarlane, [69] who has shown that the use of the ^{14}C carbonate label is to be preferred despite the greater efficiencies of incorporation into albumin with the labelled amino acid. The advantages displayed by $^{14}CO_2$ arise out of the fact that arginine production is almost if not entirely confined to the liver. [69, 107]

After the administration of ^{14}C guanidine arginine, distribution to, and labelling of many body tissue proteins occurs. The intracellular specific activity of the arginine used for the synthesis of these proteins will depend on the extent to which the incoming labelled amino acid is diluted with endogenously produced arginine. Since this dilution only occurs in the liver, precursor specific activities presented at the site of albumin synthesis will be lower than those at the extrahepatic sites of gamma globulin production. In the subsequent isolation of albumin from mixed plasma proteins, minimal degrees of contamination with gamma globulin of high specific activity may result in serious errors. [69] On the other hand the use of the ^{14}C carbonate results in albumin of higher specific activity than that of gamma globulins, since the latter will become labelled only after ^{14}C arginine produced in the liver has diffused to gamma globulin producing tissues and has become diluted with unlabelled arginine in the body water. [69] As a result errors due to contamination with gamma globulins are reduced and rigorous protein purification methods become unnecessary. Furthermore, since body tissues are not significantly labelled after carbonate administration, recycling of the label is reduced to negligible levels and in consequence the radiation exposure to which human subjects would be exposed is minimal.

The fundamental / .....

The fundamental assumption on which the ^{14}C carbonate method is based, is that both urea and albumin are synthesised from the same pool of labelled arginine and the evidence upon which this assumption rests is indirect. Whilst there is little doubt that the liver is the only site of albumin production, [112 - 114] the presence of extrahepatic arginase in brain, [115, 116] skin and kidney [117] and plasma [118] raises the possibility of significant extrahepatic urea production. A serious objection to the validity of the method would be raised if this possibility was realised. Reeve [107] has demonstrated the presence of ^{14}C urea after administering ^{14}C arginine to hepatectomised animals, and Kornberg [119] estimated that 2% of ^{14}C urea produced after bicarbonate administration in intact cats was of extrahepatic origin. From their studies in hepatectomised dogs Tavill et al [120] have confirmed the presence and activity of extrahepatic arginase. Quantitative estimation however revealed that in terms of the normal body urea pool, the amount synthesised was insignificant and amounted to less than 2% of the normal urea synthesis rate. However when $^{14}CO_2$ was administered to hepatectomised dogs, no ^{14}C urea was recovered, confirming that $^{14}CO_2$ incorporation into the urea cycle does not occur to any measurable extent in extrahepatic sites. The carbonate label is therefore once again to be preferred, for its use will minimise any errors due to extrahepatic urea production. Though albumin and urea are exclusively synthesised in the liver the possibility exists that intracellular specific activities at the respective sites of albumin and urea synthesis are different. Whether such differences exist or not is unknown, and as Tavill et al [120] have pointed out until subcellular fractionation is undertaken to determine if specific activity differences exist, the issue will remain purely speculative.

It is also necessary to make the assumption that during the period of labelling, the relative rates of urea and albumin synthesis remain constant. If these rates of synthesis were subject to variation, the situation might arise where albumin was synthesised when precursor arginine specific activity was high, and urea might be synthesised later when the precursor specific activity had fallen. Whilst there appears to be no reason to suspect rapid fluctuation in albumin synthesis [120] urea synthesis may increase rapidly after protein intake. [107, 136] For this reason it has been procedure to exclude protein from feeds for some hours prior to the start of the experi-

ment. Though the period of labelling is short, the albumin synthesis rate calculation remains dependent upon the maintenance of a steady rate of urea synthesis throughout the synthesis interval. Possible fluctuations in the fractional synthesis rate of urea during this period in which it is being measured is prevented by similarly restricting protein intake. This principle was adopted in this thesis and full details are provided under Experimental Protocol.

THE UREA SYSTEM

The procedures used [111, 120] for calculating the FSR and the maximum specific activity of urea carbon are based on the assumption that there is flash labelling of the precursor arginine pool, and that the ^{14}C urea formed from this precursor arginine is distributed as a pulse throughout a single body urea pool. After distribution, the rate at which the labelled molecules disappear is taken as the FSR of urea since the fall in radioactivities reflects the addition into the body pool of newly synthesised unlabelled urea molecules. The value obtained by extrapolation of the semilog plot of urea carbon specific activities back to zero time is taken as the theoretical maximum specific activity of urea (measured as the specific activity of the urea carbon atom). In the light of more recent work, it has become apparent that this simplified approach is in need of modification.

(i) Measurement of urea fractional synthesis rate:

It has been noticed, [111] and later confirmed, [121 - 123] that the slope of endogenously produced ^{14}C urea activities in the plasma are consistently less steep than those of prelabelled urea administered intravenously. Jones et al [121] have explained this observation on the basis of continued incorporation of the ^{14}C label into hepatic arginine when carbonate is used, and have concluded that the assumption that there is flash labelling of precursor arginine is invalid. Endogenously produced ^{14}C urea would reflect the true fractional synthesis rate of urea only if it was rapidly freed from the liver as a single pulse. Since this condition is not fulfilled, the use of the endogenously produced ^{14}C urea will result in values for the fractional synthesis rate and maximum specific activity, derived by back extra-

polation of the graph / .....

polation of the graph to zero time, to be underestimated. In order to circumvent this difficulty, it has been proposed [121, 123] that ^{13}C urea should be administered intravenously simultaneously with the ^{14}C sodium carbonate or, alternatively, pre-labelled ^{14}C urea administered prior to the carbonate study, in order to obtain the true fractional synthesis rate of urea.

(ii) Derivation of theoretical maximum specific activities of urea carbon

The more recent procedure advocated by Koj and McFarlane [123] takes cognisance of the fact that endogenously produced ^{14}C urea is delivered gradually into the body urea pool, maximum observed plasma specific activities being present after approximately 30 minutes of the administration of ^{14}C carbonate. [69, 107] This procedure, the "area method" assumes instantaneous distribution of urea throughout the body urea pool, and entails measuring the area under the ^{14}C urea specific activity curve (on linear paper) and multiplying this area by the plasma slope of simultaneously administered ^{13}C urea.

It is obviously desirable in the interests of greater accuracy to be able to implement these modifications. Unfortunately it has not been possible to do this in the experiments reported in this thesis. In the first instance facilities for the use of the stable isotope ^{13}C were not available, whilst the use of small animals (which limited the amount of blood that could be sampled) and the particular experimental design chosen made it extremely difficult to derive the additional information required with exogenous ^{14}C urea. In the light of these difficulties, the author has been obliged to derive the urea FSR and maximum specific activity from the plasma curve of endogenously produced ^{14}C urea.

There is some difference of opinion as to the magnitude of the error that may be introduced into the final albumin synthesis rate value by omitting these modifications. Koj and McFarlane [123] have reported this error to be in the order of $\pm$ 10%. On the other hand Rothschild [124] has found no significant difference between the plasma slopes of exogenous ^{14}C urea or endogenous ^{14}C urea in either fasted or fed rabbits. Jones et al [121] believed that since the argument for continued

incorporation / .....

incorporation of the precursor label almost certainly applies to albumin as well as urea, the inaccuracies inherent in adopting the flash labelling hypothesis are to an extent mutually compensating and the final error is unlikely to be of serious proportions.

It remains to be pointed out that the methods employing exogenously labelled urea for measuring the FSR and maximum specific activity of urea both assume that the plasma urea specific activity is a single exponential, reflecting the synthesis and catabolism of urea within a single body pool. The recent studies of Jones et al [121] have shown that the plasma curve in fact consists of two exponentials suggesting that labelled urea behaves in the body as if there were two compartments. At the time of writing this thesis no published reports have been received on the implications of these findings.

Observed plasma urea specific activities may be altered in two additional ways. In the first instance alterations may occur due to extrahepatic urea synthesis. This question was discussed on Page 25 where it was concluded that when the carbonate label is used, extrahepatic urea synthesis is negligible and may be disregarded as a potential source of error. The last remaining possible source of error in respect of the plasma urea specific activity slope may be an outcome of endogenous catabolism of urea in the gut by urease containing bacteria. As a result, ^{14}C urea after diffusing to the gut may be split into $^{14}CO_2$ and ammonia, the ^{14}C label recycled and used again for urea synthesis. This problem has been investigated by Regoeczi et al [109] and McFarlane et al [111] who have shown that whereas extensive recycling of the label occurs with ^{15}N urea, recycling of the ^{14}C label after ^{14}C urea administration is negligible, the latter being eliminated via the lungs. Whilst treatment with Neomycin reduced the amount of urea catabolised in the gut, such treatment had no effect on the fractional rate of urea synthesis [109] when the ^{14}C label was used. One may conclude again that endogenous catabolism of urea does not introduce significant error into the method when a ^{14}C label is used.

THE ALBUMIN SYSTEM

Losses of intravascular ^{14}C albumin resulting from catabolism and diffusion into the

extravascular / .....

extravascular spaces have to be measured in order to obtain the theoretical maximum specific activity of albumin, (measured as the specific activity of the guanidine carbon atom). Whereas ^{14}C urea may be detected in the plasma within a few minutes after ^{14}C carbonate injection, the first appearance of ^{14}C albumin occurs 30 - 60 minutes later, [69, 120, 123] the delay representing the time taken for newly synthesised albumin molecules to be transported from the sites of synthesis on the endoplasmic reticulum to the blood stream. [125, 126]

Loss of ^{14}C albumin may be measured by making reference to the disappearance of a pulse of ^{125} or ^{131} I labelled albumin injected simultaneously with the ^{14}C carbonate, making due allowance for the gradual delivery of ^{14}C albumin into the plasma. The use of an intravenous dose of iodinated albumin rests on two assumptions, firstly, that ^{14}C albumin is also delivered directly to the intravascular compartment and not via the lymph channels, and secondly that iodinated albumin may be regarded as representative of the behaviour of ^{14}C albumin. The first assumption has been validated by the studies of Smallwood et al [125] and the second assumption has been proven in a number of studies previously referred to in which the "in vivo" behaviour of ^{14}C and iodine labelled albumin was compared. [65, 85 - 87] The area method which has been suggested for measuring ^{14}C urea losses, is on theoretical grounds not suitable for correcting albumin ^{14}C losses because of the multi-exponential nature of the iodinated albumin plasma curve. [123] The strength of this objection has been assessed by Koj and McFarlane [123] with the use of digital and analogue computers, and on the basis of their findings it was concluded that for synthesis intervals of the order of 200 - 300 minutes, satisfactory accuracy was still achieved when an area method was used. The method requires the time course of the appearance of ^{14}C albumin to be determined and the integrated guanidine carbon radioactivities in the plasma to be multiplied by the fractional rates of loss from the plasma, the latter derived by graphic analysis of the iodinated albumin plasma curve.

In this thesis practical considerations have governed the choice of a more elementary method of correcting for ^{14}C albumin losses. Whilst there was no reason to suspect that the time of arrival of ^{14}C albumin in the plasma would vary amongst the different groups of experimental animals, (this depending on transport factors alone)

it was quite / .....

it was quite conceivable that the area of the ^{14}C albumin plasma curve might vary, not only between the different groups of animals, but also within each group at the beginning and end of the study period. The accurate definition of the time course of ^{14}C albumin under these varying conditions would have been prohibitive in terms of both time and expense, particularly since a single rat would not have provided sufficient plasma for the description of the curve in its entirety, and the curve would have had to be derived from the plasmas of a number of rats. In addition the accurate definition of the iodinated albumin plasma curve throughout the six hour synthesis rate period would have greatly increased the volume of blood sampled, possibly resulting in altered glomerular filtration rates and upsetting the steady state conditions with respect to urea metabolism.

The procedure adopted therefore was to compare ^{125}I or ^{131}I albumin radioactivities in similar aliquots of plasma at zero time and again at the end of the synthesis rate period as has been the procedure elsewhere. (120, 124, 127, 137) The observed ^{14}C albumin specific activity at the end of the synthesis rate interval was multiplied by the amount which the iodinated albumin had been observed to decrease. In using this approach the author is aware that allowance has not been made for the gradual arrival of ^{14}C albumin in the plasma.

A final point concerning the time courses of urea and albumin in the blood requires to be mentioned. Since albumin molecules only appear in the blood after 30 minutes of injecting the carbonate, hypothetical albumin specific activity at t_o should be compared in the synthesis rate formula with similar urea specific activities that would have existed at t_o - 30 minutes. The magnitude of the error associated with omitting this additional refinement is small and the correction may be omitted entirely. (123)

Because the validity of the carbonate method is not dependent on the existence of prolonged steady states, it is well adapted for use in metabolically unsteady conditions. It also offers the means whereby albumin synthesis may be measured independently of albumin catabolic rate, the former method studying the incorporation of ^{14}C into albumin, and the latter the elimination of iodine from albumin. This

statement requires /.....

statement requires a minor qualification since the synthesis rate method is connected with catabolism insofar as catabolism will account for some of the losses of ^{14}C albumin molecules, which will require to be assessed and corrected for.

Synthesis and catabolic rates as measured by these two methods have been compared under steady state conditions and satisfactory agreement has been achieved in a number of studies. (69, 106, 120, 127, 128) Since the synthesis rate is measured over a short period of hours, and the catabolic rate represents the average value obtained over a number of days, it seems unlikely under these circumstances that better agreement could be expected. The newer techniques described (129) for the short term measurement of the catabolic rate seems to hold greater promise of effecting better agreement between synthesis and catabolic rates.

Indirect Methods of Measuring Albumin Synthesis

The experiments reported in this thesis represent the first direct measurement of albumin synthesis after hypophysectomy and growth hormone replacement therapy after hypophysectomy. Since it will be necessary to compare the present data with that obtained by previous workers, some of the indirect methods by means of which these investigators measured albumin synthesis require to be mentioned.

By measuring the total amount of albumin catabolised over an experimental period and comparing this with the change in the total albumin pool in the body over the same period, an indication is obtained of the amount by which the synthesis rate differs from the catabolic rate, and this approach has been used by Rothschild (130) to determine the extent to which increased synthesis had offset the effects of experimentally induced hypercatabolism. Other workers (57, 131, 132) have based synthesis rate measurements on the plasma albumin specific activity curve, on the assumption that the falling specific activity reflects only the rate of addition of newly synthesised unlabelled molecules to the plasma. This approach is of doubtful value since, as discussed previously, plasma specific activities are complicated by the additional contribution of albumin of higher specific activity returning from the extravascular

compartments, which in unsteady states may occur at varying rates. A more useful procedure was proposed by Matthews [101] which gave a combined value for albumin synthesis and transfer of albumin molecules from the extravascular compartment. Whilst the individual contributions to the combined "synthesis plus transfer rate" could not be determined, approximate values could be obtained by means of an analogue computer. [133]

CHAPTER THREE

PART 1. MATERIALS AND METHODS

ANIMALS

Male albino rats of the Wistar strain bred at this Medical School, and weighing between 230 g. and 260 g. were chosen for investigation. The rats were housed in single cages, and fed a standard rat cube diet (Vereeniging Consolidated Mills Limited, Cape Town) containing 20% mixed protein.

SURGICAL PROCEDURES:

(1) Hypophysectomy was performed under ether anaesthesia by the author via the parapharyngeal route.[60] The trachea was intubated with a fine polythene tube and the base of the skull drilled with an electric powered dental drill. The gland was aspirated into a finely drawn out glass pipette and in all cases the three pituitary lobes were identified in the glass pipette after aspiration. By this means a complete hypophysectomy was assured since experience had shown that the lobes themselves do not fragment. In addition the pituitary fossa was carefully checked at post mortem after the experiment for complete removal of the gland. Full details of the operative technique are provided in the appendix.

(2) Sham operation. The sham operation carried out was identical in all respects to the hypophysectomy, save that the pituitary gland was left intact after drilling the base of the skull.

MATERIALS:

(i) ^{131}Iodine and ^{125} iodine were obtained from the Radio-Chemical Centre, Amersham, (Bucks. U.K.), as carrier free preparations, free from reducing agent.

(ii) ^{14}C Sodium carbonate (S.A. 20 - 40 mC/m-mole) was obtained from Phillips-Duphar, Holland as an aqueous solution. This preparation was diluted with normal saline to provide a concentration of 0.5mC/ml, and stored in sealed sterile pyrex tubes until used.

(iii) Bovine Growth / .....

(iii) Bovine Growth Hormone (NIH- GH- B14). The highly purified hormone was supplied by the Endocrinology Study Section of the National Institutes of Health and had a mean potency of 1.04 U.S.P. units per mg. Solution was achieved at pH 9 by the addition of 0.1 ml. of normal saline, and 0.05 ml. of 0.1 N NaOH per mg. of hormone. The volume was then adjusted with normal saline, so that the daily dose of 0.25 mg. could be delivered subcutaneously in 0.5 ml. The characteristics of this hormone preparation as determined by the National Institutes of Health are presented in full in the appendix.

PLASMA ALBUMIN CONCENTRATION:

Plasma albumin concentration was determined by the method of Fernandez et al [67] in which after the preliminary removal of globulins with HCl-ethanol, albumin was precipitated with 0.2M sodium acetate in absolute ethanol, and quantitated colorimetrically with Biuret reagent. Samples were read after thirty minutes against a known standard of bovine albumin on a Zeiss PM Q11 spectrophotometer at 545μ. Since available samples of plasma were frequently small in volume, the method was scaled down for use on half volumes.

ELECTROPHORESIS:

Cellulose acetate electrophoresis was carried out in 0.05M veronal buffer (pH 8.6). A current strength of 0.3mA per centimeter width of strip was applied for one and a half hours. Staining and clearing of the strips was achieved by immersion in 0.2% (w/v) Ponceau S stain and 10% (v/v) acetic acid respectively.

PLASMA UREA CONCENTRATION:

Plasma urea concentration was determined on a Technicon autoanalyser as described by Marsh et al. [139]

PREPARATION OF RAT ALBUMIN:

Globulins were precipitated from plasma with 50% saturated ammonium sulphate at 37°. [64] Albumin was precipitated from the supernatant fluid by

adjusting / .....

adjusting the pH to 4.5 with 10% (v/v) acetic acid. The supernatant fluid was discarded and the albumin precipitate redissolved in distilled water. To remove any residual salt the sample was dialysed overnight at 4°C against distilled water. After dialysis the concentration was adjusted to 3 g% (w/v), and its homogenecity checked by cellulose acetate electrophoresis. Generally 5% of contaminating globulins were found to be present by this method of preparation.

PREPARATION OF ^{125}I OR ^{131}I LABELLED RAT ALBUMIN

Iodination of rat albumin was performed by the iodine monochloride method, (76) and full details are provided in the appendix of methods. A mean level of substitution of less than one atom of radioiodine per albumin molecule was achieved.

(i) For catabolic rate studies ^{131}I was used. After iodination one ml. of fresh rat plasma was added to the iodinated albumin to prevent self irradiation damage. This was followed by ammonium sulphate precipitation and dialysis as described above. In this way free ^{131}iodide, residual salt, and any contaminating labelled globulins were effectively removed. Trichloroacetic acid precipitation on the final preparation showed that greater than 99% of the radioactivity was protein bound. The iodinated sample was subjected to cellulose acetate electrophoresis and the radioactivity was shown to reside only in the albumin fraction. Normal saline was added so that approximately 20 μc of ^{131}I albumin was contained in 1 ml., after which sterilisation was achieved by "millipore" filtration.

(ii) For synthesis rate measurements, albumin was labelled with either ^{125}I or ^{131}I. Free radio iodide was removed by passing the sample through a column of Deacidite resin in the chloride form after which 99% of the radioactivity was shown to be precipitable with trichloracetic acid. The sample was diluted with normal saline and the ^{14}C sodium carbonate solution, to provide doses of iodinated albumin of approximately 4 μC/ml., and then sterilised by "millipore" filtration.

PLASMA VOLUME:

This was determined by isotope dilution using ^{131}I or ^{125}I labelled rat albumin.

RAT GROWTH HORMONE:

In order to determine the time taken for endogenous growth hormone to disappear completely from the body, rat growth hormone (RGH) was measured by radioimmunoassay in a randomly selected group of animals for nine days after hypophysectomy. Highly purified R.G.H. was iodinated by the method of Hunter and Greenwood [74] and in addition served as reference standard. Monkey anti R.G.H. antibody was used in the assay, and separation of antibody-bound and free ^{131}I R.G.H. complexes was achieved by chromato-electrophoresis. [140]

PLASMA CORTICOSTERONE: *

Total 11 hydroxycorticoids in the plasma were measured by the method of Mattingly. [141] Since in the rat the ratio of corticosterone to cortisol is approximately 7:1, total 11 hydroxycorticoids are hereafter for convenience referred to as plasma corticosterone.

MEASUREMENT OF ALBUMIN CATABOLIC RATE

Thyroidal uptake of radio iodine released by catabolism was prevented by the provision of 0.005% (w/v) sodium iodide in the drinking water for 48 hours prior to, and for the duration of all catabolic rate studies. Urinary excretion of the label was measured indirectly by means of the daily fall in whole body gamma radiation.

Approximately 20 μC of ^{131}I labelled rat albumin was administered intravenously into a tail vein under light ether anaesthesia. Total body activity was assayed immediately, and ten minutes after injection, the rats were bled from the tip of the tail for plasma volume, and 'Day O' plasma albumin specific activity determinations. Whole body activity and plasma albumin specific activity was determined daily at the same time for the duration of the experimental period.

Whole body activity was measured in a small whole body counter consisting

* Measured in the Department of Chemical Pathology with the assistance of Dr K. Arntsen.

of a ring of six / .....

of a ring of six matched G26 Pb Geiger-Muller tubes. Pulses were fed via a Probe Unit (Ekco type N558) and recorded on an Ekco scaler (type N530G). The probe unit served to impose a constant Dead Time of 200 μ sec. on all pulses, thus facilitating accurate Dead Time correction over a wide range of observed counts. Observed counts were corrected for Dead Time by the formula:-

$$N\ (\text{corrected}) = \frac{N\ (\text{observed})}{1 - N\ (\text{observed}) \times \lambda} \quad \text{where } \lambda = \text{Dead Time.}$$

Dead Time corrections were neglected once the whole body activity fell below 150 cps.

Plasma activity was assayed on small aliquots (0.02 ml.) of plasma in a Packard Autogamma Spectrometer. Sampling errors were minimised by the use of solid anticoagulant and constriction micropipettes. Plasma was also obtained daily for the determination of the albumin concentration. After correction for decay by means of appropriately made up standards, and after Dead Time corrections, the daily absolute and fractional catabolic rates were calculated from the following formulae:-

$$\text{Absolute CR (mg/day)}^{(101)} = \frac{\text{fall in whole body activity on Day n } (\mu C/\text{day})}{\text{mean plasma albumin specific activity on Day n } (\mu C/\text{mg albumin})}$$

$$\text{Fractional catabolic rate (\% intravascular pool/day)} = \frac{\text{fall in whole body activity on Day n}}{\text{mean plasma activity on Day n}} \times 100$$

MEASUREMENT OF ALBUMIN SYNTHESIS RATE:

Albumin synthesis was measured over a six hour period. In the normal control rats, and in those experimental rats eating ad libitum, albumin synthesis rate was measured after a 10 - 12 hour fast. In the pair fed rats, the quantity of food provided on the preceding day was often rapidly consumed. In this instance the synthesis rate measurement was preceded by a fast of 12 - 16 hours. In all cases food was withheld during the six hour period of measurement but free access to drinking water was permitted at all times.

Under light ether / .....

Under light ether anaesthesia approximately 200 μC of ^{14}C sodium carbonate, and 2 μC of ^{125}I or ^{131}I labelled rat albumin was administered simultaneously by intravenous injection at the base of the tail. Ten minutes later the rats were bled from the tip of the tail for determination of the plasma volume. Half hourly, from hours three to six, small (0.1 ml.) samples of blood were obtained from the tip of the tail for the determination of urea carbon specific activities. At six hours the rats were lightly anaesthetised with ether and exsanguinated by cannulating the aorta. Albumin guanidine carbon specific activity, and albumin and urea concentrations were determined on this final sample of plasma. Aliquots of plasma from the ten minute and final bleedings were counted in order to provide the correction factor for the albumin guanidine carbon specific activity, as explained in Chapter 2.

Once post mortem examination revealed the absence of pituitary gland tissue the appropriate blood and plasma samples from four rats were pooled prior to processing. Full details of the laboratory methods and the calculations required for the determination of the albumin synthesis rate are provided in the Appendix of Methods.

In broad outline, the laboratory procedure of measuring albumin synthesis depends on the enzymatic conversion of urea carbon to CO_2 with urease. The volume of CO_2 liberated is measured in a calibrated manometer and then trapped in a phenylethylamine-methanol mixture which in turn is transferred to a liquid scintillation mixture and assayed for radioactivity. Calibration of the manometer with known standard urea solutions makes it possible to convert volumes of CO_2 to mg. C, and, after radioactive assay, to express urea carbon specific activities as C.P.M./mg. C. Since the volumes of enzymatically produced CO_2 are small it is necessary to undertake their measurement on a high vacuum gas-train. Working pressures of 1 to 3×10^{-3} mm.Hg. were achieved by a two stage high vacuum pump. By differential freezing with liquid nitrogen and solid CO_2-ethanol, the CO_2 sample may be further freed from any contaminating gasses remaining in the system. The high vacuum gas-train used was designed and constructed locally by Hoffenberg [142] and is depicted in the appendix.

For urea carbon / .....

For urea carbon specific activity measurement, samples of whole blood were first deproteinised and then incubated with urease. The volume of liberated $^{14}CO_2$ was measured manometrically on the high vacuum gas-train as outlined above, and then condensed into phenylethylamine-methanol [143] at the temperature of liquid nitrogen. After thawing, the phenylethylamine was transferred quantitatively to a scintillation mixture and counted in a Beckman Liquid Scintillation Counter.

Albumin guanidine carbon specific activity was measured on albumin extracted from plasma by the method of Korner and Debro. [66] Cellulose acetate electrophoresis consistently demonstrated the absence of contaminating globulins after the extraction processes. The protein was subjected to acid hydrolysis and the resultant amino acid residue incubated with arginase thus splitting off the guanidine group to yield urea. This urea was processed for specific activity measurement in identical fashion to that described above.

The slope of the semi log plot of the falling urea carbon specific activities from blood sampled over the three hours provided the fractional synthesis rate of urea, and back extrapolation of the graph to zero time (t_o) gave the theoretical maximum specific activity of urea carbon. In order to obtain the maximum (t_o) specific activity of albumin guanidine carbon, the specific activity observed at six hours was increased by a variable factor derived from the disappearance (from the intravascular compartment) of the iodinated albumin administered simultaneously with the ^{14}C sodium carbonate at the beginning of the experiment. Fractional and absolute synthesis rates were then calculated as follows:-

Fractional synthesis rate albumin (% IVP/hour) =

$$\frac{\text{fractional S.R. urea (\%/hour)} \times \text{S.A. Albumin to}}{\text{S.A. urea } t_o}$$

Absolute S.R. (mg/hr.) = fractional S.R. x I.V.P.

where I.V.P. = albumin concentration x plasma volume.

These hourly values were multiplied by twenty-four to provide an average daily value.

Part 2 / .....

PART 2. EXPERIMENTAL PROTOCOL

The object of this thesis was to ascertain what role, if any, growth hormone has in regulating albumin metabolism. Albumin synthesis and catabolic rates have therefore been measured under conditions of growth hormone deficiency, and after growth hormone replacement therapy. It was decided to conduct the investigation in rats firstly because it was anticipated that large numbers of animals would be required, and secondly because hypophysectomy is most easily performed and carries the best chance of success in this animal. Since growth hormone is not a trophic hormone i.e. it does not act via a target endocrine organ, growth hormone deficiency can only be produced by anterior hypophysectomy. However, in order to avoid confusion when the pituitary fossae were examined at post mortem for gland remnants, it was decided to perform a total (anterior and posterior) hypophysectomy.

Since rat growth hormone is not commercially available, replacement therapy was achieved by using bovine growth hormone supplied in pure form by the National Institute of Health. Since the hormone derives from a higher species it is biologically active in the rat. (144) The dose of 0.25 mg/day was chosen empirically to correspond roughly with that used by other workers. Ideally the replacement dose chosen should be aimed at providing physiological replacement, but in the absence of any data to suggest a comparable amount of bovine growth hormone to replace rat growth hormone, the dose of 0.25 mg/day was selected and would appear to be suitable since clearcut effects on many aspects of intermediary metabolism are achieved at this dosage level. (32) Growth hormone injections were commenced twenty four hours before hypophysectomy and given daily until twenty four hours before the synthesis rate experiments. Given in this way growth hormone effects would cover the operative period (174) and steady state conditions with respect to urea production would not be jeopardized by growth hormone administered shortly before the synthesis rate measurement. (174)

In view of the short half life of growth hormone, (145, 146) it was anticipated that the effects of growth hormone deficiency would manifiest rapidly after hypophysectomy and for this reason the study was confined to the first nine days after hypophysectomy.

The presence / .....

The presence of anorexia in the hypophysectomised rat necessitated the inclusion of an additional group of control rats in which this factor was assessed. This problem could be tackled either by force feeding hypophysectomised rats, or, by restricting the food intake of intact rats. On theoretical grounds force feeding hypophysectomised rats is to be preferred but this technique (requiring the passage of a stomach tube) is at the best of times a traumatic procedure, and it was felt that in rats just recovering from the effects of surgery the additional manipulations involved would not only prevent wound healing, but severely prejudice their chances of survival. In the light of these difficulties it was decided to pair feed intact rats with hypophysectomised rats eating ad libitum. The effect of surgical intervention per se, was assessed by subjecting these rats to a sham operation.

The experiments were designed as follows:-

Albumin Catabolic Rate Studies

Albumin catabolic rate was measured in four groups of rats

1 Control group (16 rats)

11 Experimental groups

(a) Hypophysectomised group (9 rats)

(b) Pair fed sham operated group (5 rats)

(c) Hypophysectomised group receiving growth hormone replacement therapy (9 rats)

The sixteen normal control rats were studied over a period of eight to ten days. The catabolic rate of each individual rat was determined on a daily basis, the daily values then being meaned to provide an average value for the ten day period. The average values for each of the sixteen rats were then meaned to give an overall normal value for albumin catabolism.

In the three experimental groups of rats, the tracer dose of albumin was injected and the pre-operative catabolic rate first established on a daily basis

over a period of / .....

over a period of three or four days. These daily values were meaned to obtain a single average preoperative value. Hypophysectomy or sham operation was then carried out and the fractional and absolute catabolic rates determined daily for nine days thereafter. This approach not only made it possible for the rats to act as their own controls both before the operation and throughout the nine day study period, but also made it possible to check on the quality of the tracer dose by inspecting the pre-operative catabolic rate as explained in chapter 2.

Albumin Synthesis Rate Studies

The large amounts of plasma or blood required, and the need for complete elimination of circulating isotopes precluded serial synthesis rate determinations in the same rats as was possible when the catabolic rate was measured. Equal aliquots of blood or plasma from four rats was therefore pooled to provide each separate synthesis rate value.

Normal control values were obtained on nine such batches of rats (36 individual rats). In the three experimental groups (as above) the synthesis rate was determined on Days 2, 4, 6 and 9 post-operatively. On Days 4 and 6 the synthesis rate was measured in two batches of rats, but in order to permit statistical comparisons between observations made at the beginning and end of the study period, a total of six batches were studied on Days 2 and 9.

CHAPTER FOUR

RESULTS

1. FOOD CONSUMPTION

The average quantities of food consumed daily by the normal control rats, and by the three experimental groups of rats, are presented below in figure 4:1. Normal values were derived from forty rats observed for a period of not less than two weeks. In respect of the quantities of food eaten by the three experimental groups of rats, the following features require to be noted.

(i) At all times the daily intake of these groups is well below the average normal intake.

(ii) As the hypophysectomised rats (eating ad libitum) recovered from the effects of the operation, larger quantities of food were consumed. The values depicted in figure 4:1 were derived from twenty-one rats observed daily for the nine day study period.

(iii) The sham-operated group of rats, which were pair fed to the hypophysectomised rats, made a swift recovery from the effects of the operation and it was clear from the eagerness with which the daily rations of food were consumed that their normal appetite was retained. The daily food ration was always completely consumed so that the graphs for this group of rats and the hypophysectomised rats coincide exactly.

(iv) Bovine growth hormone appears to have no significant effect on the anorexia present after hypophysectomy since quantities of food eaten by the growth hormone treated rats eating ad libitum were of the same order as the untreated hypophysectomised rats. Here again this conclusion is based on observations made on twenty-one rats treated with growth hormone for nine successive days post-operatively.

2. DISAPPEARANCE OF PITUITARY HORMONES AFTER HYPOPHYSECTOMY

A successful hypophysectomy was readily evident from the precipitous and persistent weight loss, anorexia and lethargy displayed by the rats after the operation.

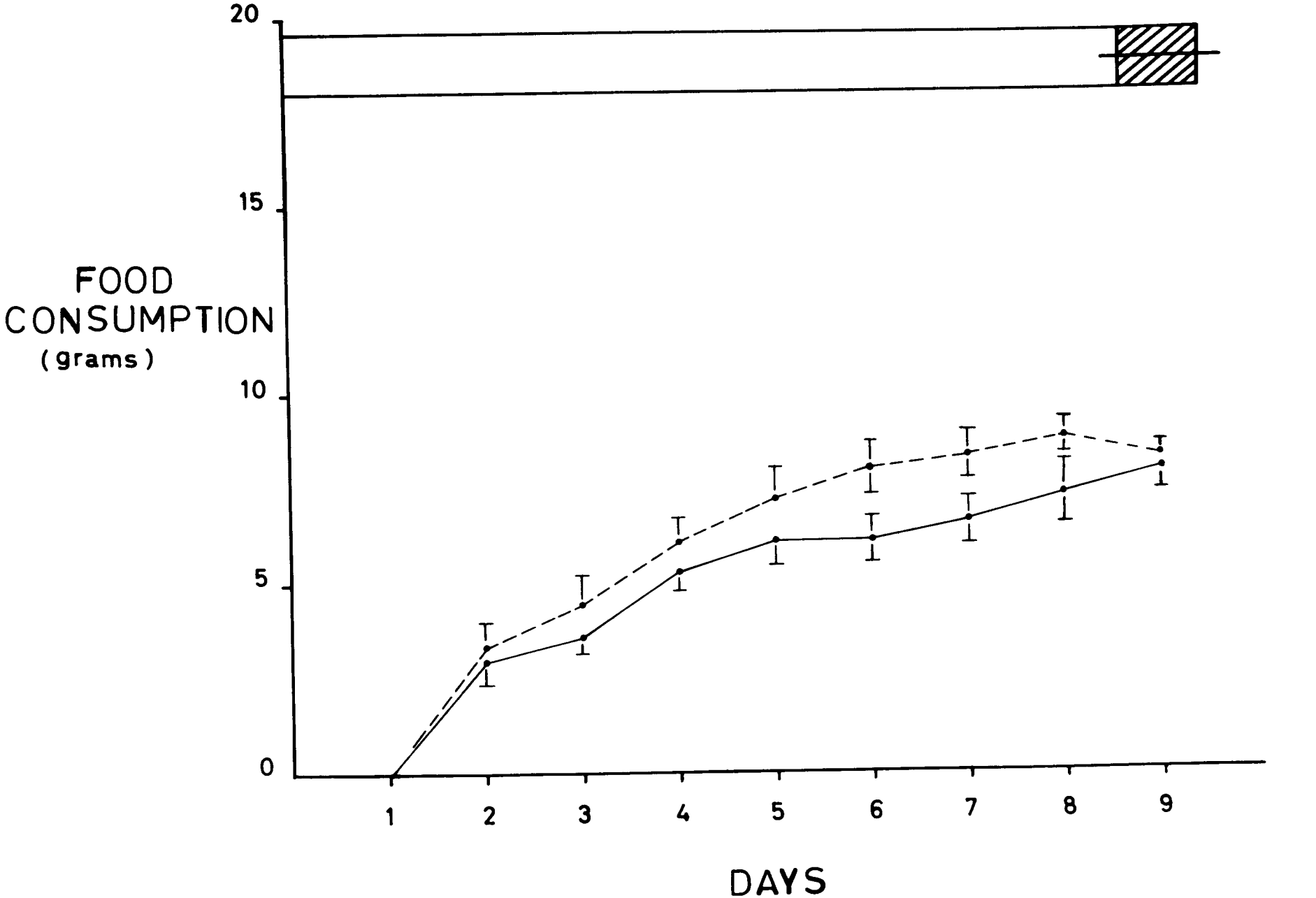

Figure 4:1 Post operative food consumption. The hatched area is the mean $\pm$ Standard Error of the intake in the normal control rats. Solid line = Combined graph for both hypophysectomised and sham operated pair fed rats. Interrupted line = graph for hypophysectomised rats treated with growth hormone. Results expressed as the mean $\pm$ Standard Error.

Biochemical confirmation of hypophysectomy was obtained in a small series of rats displaying the clinical features of hypophysectomy by measuring plasma growth hormone and corticosterone levels on days 2, 4, 6 and 9 postoperatively. Both these hormones disappeared rapidly from the plasma, and in eight rats shown at post-mortem to be successfully hypophysectomised, neither hormone was detectable in the plasma forty-eight hours after the operation. The possible implications of these findings will be discussed in chapter five.

3. ALBUMIN METABOLISM

(a) Control group

The plasma albumin half-lives and rates of synthesis and catabolism obtained in the normal control rats are presented in Table A and are compared with those values obtained by other workers. In general there is satisfactory agreement suggesting that the albumin tracer used in the present study was biologically acceptable and that the values obtained represent the normal range in the rat.

Table A / .....

TABLE A

NORMAL RAT VALUES

AUTHOR		METHOD
1. Fleck and Munro H.N. (138)	$T_{\frac{1}{2}}$ = 2.8 days	^{131}I albumin
2. Cohen S. (216)	$T_{\frac{1}{2}}$ = 2.8 days F.C.R. = 73% I.V.P./day	^{131}I albumin
3. Freeman T. and Gordon A.H. (166)	$T_{\frac{1}{2}}$ = 2.7 days F.C.R. = 76% I.V.P./day	^{131}I albumin
4. Kirsch et al (127)	A.C.R. = 268 mg/day/300 g. rat A.S.R. = 280 mg/day/300 g. rat	^{131}I albumin ^{14}C carbonate
5. Present study	$T_{\frac{1}{2}}$ = 2.7 days F.C.R. = 77.6% I.V.P./day A.C.R. = 207 mg/day/240 g. rat*	^{131}I albumin
	F.S.R. = 73.5% I.V.P./day A.S.R. = 200 mg/day/240 g. rat*	^{14}C carbonate

*range = 230 g - 250 g

I.V.P. = intravascular pool
A.C.R. = absolute catabolic rate
F.C.R. = fractional catabolic rate
A.S.R. = absolute synthesis rate
F.S.R. = fractional synthesis rate

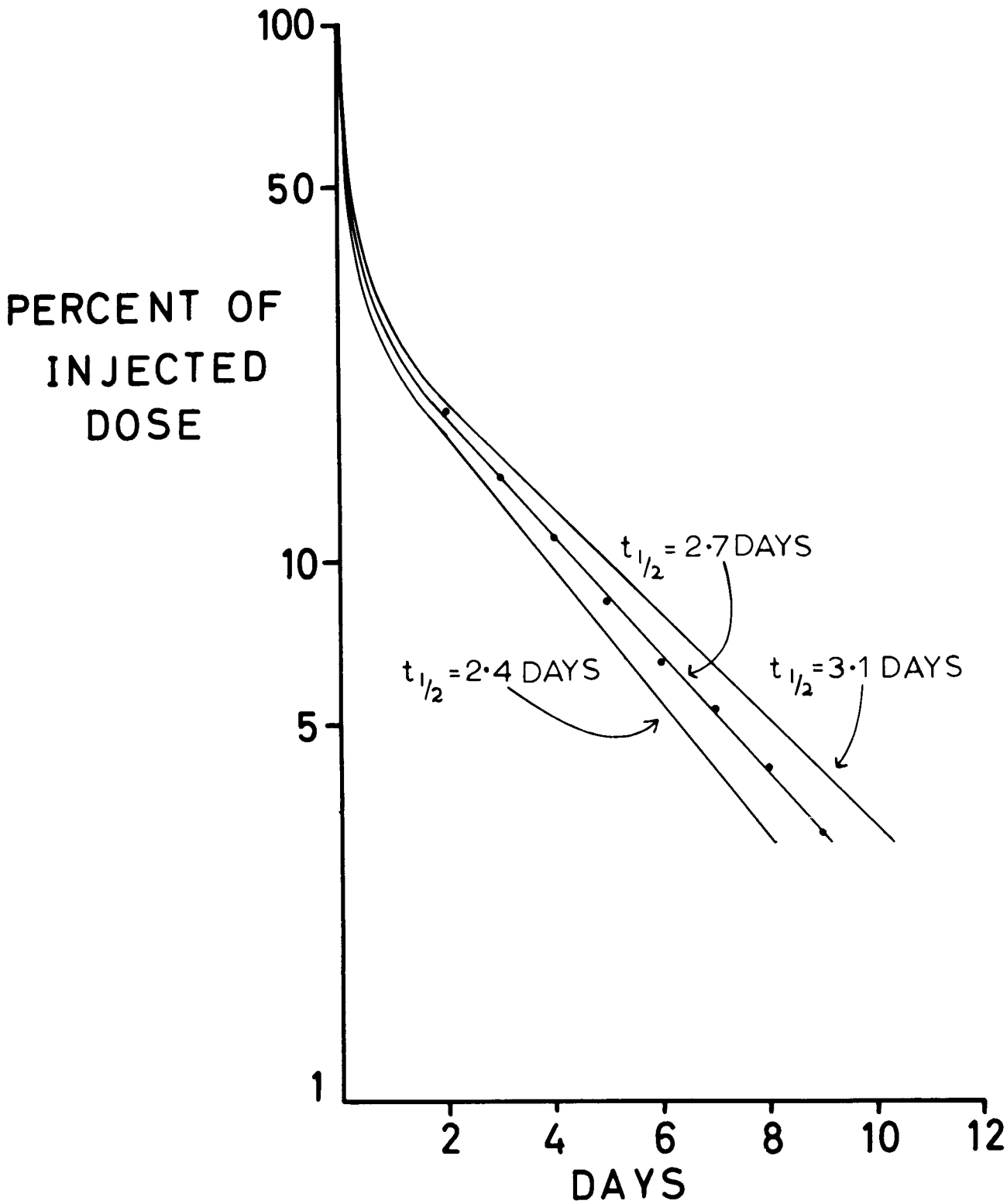

Figure 4:2 Plasma albumin half life.

Figure 4:2 displays the normal values for plasma albumin half-life. The daily plasma activities of the sixteen rats (expressed as a percent of initial plasma activity) were meaned and plotted on log linear paper, and the best fitting line for those points after forty-eight hours plotted by the method of least mean squares. The mean plasma albumin half-life was 2.7 days with a standard deviation of 0.2 days. The range of 2.4 to 3.1 days is also depicted in the diagram.

It has not been the express intention of this thesis to evaluate the agreement between steady state measurements of albumin synthesis and catabolism by the two independent methods used. Since in most instances synthesis and catabolic rates were not measured in the same rat, there is insufficient data to provide a measure of correlation between the rates of these two processes as measured by the two different techniques. The normal values obtained for synthesis and catabolism are displayed in table A.

(b) Experimental groups

The effects of hypophysectomy, sham operation and pair feeding, and hypophysectomy plus growth hormone treatment on albumin metabolism are presented in both diagramatic and tabular form, (Figures 4:3 and 4:4, and Tables B to E).

Since values for absolute synthesis and catabolic rates will partly depend on the size of the intravascular pool, comparisons between various absolute values could be misleading if the animals were of different sizes.

Despite the greatest care in grouping rats of comparable body weight, plasma volumes varied as a result of the surgical and dietary manipulations. Absolute rates were not expressed in terms of body weight, since correction for change in body weight alone does not take into account the marked differences between the body composition of starved, hypophysectomised, and growth hormone treated animals [30, 36, 37] upon which the plasma volume is to a large extent dependent. Animals of the same weight may therefore have plasma volumes that differ considerably. Fractional rates of synthesis and catabolism not being affected by such changes have therefore also been presented.

In figures 4:3 and 4:4 the hatched areas represent the mean $\pm$ 2 standard

deviations of the / .....

deviations of the values obtained from the normal control rats. Each synthesis rate value is derived from the pooled plasmas of four rats. The daily catabolic rate values for the three experimental groups of rats have been charted as the mean $\pm$ 1 standard deviation. The values presented in figures 4:3 and 4:4 for the albumin concentration were those obtained in the catabolic rate studies, and these are also charted as the mean $\pm$ 1 standard deviation. For the sake of clarity the albumin concentrations of the rats in which the synthesis rate was measured have been omitted from these diagrams, and are presented together with the synthesis rate data in Table B. The catabolic rate data is presented in Tables C, D and E.

Note: Values for the absolute catabolic rate and albumin concentration on days 4, 6 and 8 in the untreated hypophysectomised rats are not available. Early in this study it was felt that daily sampling might increase the post hypophysectomy mortality rate. In an attempt to reduce the amount of blood sampled, on these days sufficient blood was obtained for the calculation of the fractional catabolic rate alone, but not the absolute catabolic rate nor albumin concentration. As experience was gained this precaution was found to be unnecessary and was dispensed with, so that complete data is available for the growth hormone treated hypophysectomised rats.

For the interpretation of the data a number of comparisons both within and between the various groups were undertaken and evaluated statistically, The following comparisons were made:-

(1) Fractional and absolute synthesis rates on days 2 and 9 in the three experimental groups were compared with normal values (Table V).

(2) Fractional and absolute catabolic rates in the three experimental groups were compared with normal values. No table is presented but the relevant statistical data is included in the text.

(3) In Table VI the synthesis rates on Days 2 and 9 post-operatively were compared with the catabolic rates on the same days. Comparison is made within each experimental group only.

(4) The absolute and fractional synthesis rates of each experimental group on Days 2 and 9 post-operatively were compared with those of the remaining

two experimental / .....

two experimental groups on the same days. The data is presented in Tables I and II respectively.

(5) The absolute and fractional catabolic rates of each experimental group on days 2 and 9 post-operatively were compared with those of the remaining two experimental groups on the same days. The data is presented in Tables III and IV, respectively.

The significance of differences between observed values was tested by the Mann-Whitney 'U' test. Wherever possible, exact values of P are provided. Where sample sizes precluded this, critical values of 'U' are provided at the .001 and .01 significance levels. Where the observed value of 'U' is less than or equal to the critical value of 'U' then the difference between the two populations is significant at that significance level. The following convention was adopted in expressing values of 'U' :

$$\text{'U' observed} \; . \; \frac{\text{Critical 'U' at .001 level}}{\text{Critical 'U' at .01 level}} \quad \text{e.g. } 4\,{}^{3}_{9}$$

The two populations that were compared are significantly different at the .01 level only, since 4 is less than 9 but greater than 3.

$p \leq .05$ - probably significant; $p \leq .01$ significant;
$p \leq .001$ highly significant.

Full details of the statistical methods is provided in the appendix.

Table B / .....

		Alb. conc. g%	P.V. ml.	I.V.P. mg.	F.S.R. %I.V.P./day	A.S.R. mg/day
Normal values		2.47	11.43	282.3	74.4	210.0
		2.63	9.95	240.6	67.4	162.2
		2.24	11.01	246.6	82.3	202.8
		2.54	11.80	299.7	76.5	229.4
		2.43	11.35	275.5	75.3	207.6
		2.88	9.97	287.1	72.4	208.0
		2.80	10.38	290.5	69.8	202.8
		2.57	10.28	264.1	70.3	185.7
		2.54	10.05	255.5	73.6	188.1
				Mean	73.5	199.6
				2 S.D.	8.7	37.9
Hypophysectomy	Day 2	1.75	9.25	161.8	51.6	83.5
		1.61	10.34	166.4	33.6	55.9
		1.96	8.03	157.3	59.5	93.6
		2.01	9.06	182.1	47.5	86.4
		2.10	8.77	185.4	44.4	82.0
		2.07	8.35	173.2	40.3	69.6
	4	1.58	9.54	150.7	49.4	74.4
		1.94	8.57	166.2	41.0	68.1
	6	1.75	9.65	168.8	55.9	94.3
		1.78	8.94	159.1	30.2	48.0
	9	1.50	6.84	102.6	28.5	29.2
		1.57	10.56*	165.7	21.8	36.2
		2.12	9.87*	209.3	31.6	66.2
		1.73	6.65	116.6	37.9	44.1
		1.57	6.29	99.5	25.4	25.2
		1.98	6.12	121.8	39.1	47.5
Sham Operation and Pair Feeding	Day 2	2.42	11.92	290.8	54.9	159.6
		2.58	10.30	268.2	58.5	156.9
		2.17	8.43	183.3	64.5	118.3
		2.14	9.07	195.2	82.3	160.5
		2.32	9.11	212.3	47.7	101.2
		2.69	8.51	229.5	49.6	114.0
	4	2.21	11.21	247.7	46.8	115.9
		2.53	10.07	254.7	48.0	122.1
	6	1.65	9.56	158.0	66.7	105.3
		2.21	11.22	246.4	40.8	100.3
	9	2.42	9.84	240.0	51.1	122.6
		2.11	11.68	246.4	60.4	149.0
		2.21	8.38	185.2	66.0	114.0
		2.32	9.10	211.1	62.6	132.0
		2.11	8.94	187.8	81.8	153.1
		2.33	9.05	209.1	77.2	161.5
Hypophysectomy plus Growth Hormone	Day 2	1.56	9.73	152.4	57.3	87.3
		2.14	10.35	222.2	66.9	148.5
		1.91	9.05	172.6	71.2	122.8
		1.98	9.36	184.4	53.7	99.1
		2.06	8.72	179.3	64.0	114.7
		1.89	10.05	191.2	54.2	103.6
	4	1.85	10.28	190.1	71.5	135.8
		1.69	10.32	173.4	60.7	105.1
	6	1.60	9.67	154.7	57.1	88.3
		1.56	10.94	171.1	66.7	114.0
	9	1.64	10.66	175.2	45.8	80.1
		1.59	11.04	172.9	54.9	94.8
		1.99	10.78	213.8	44.4	94.8
		1.81	9.80	150.4	40.8	73.4
		1.77	9.15	163.8	36.0	58.8
		1.89	8.77	165.8	48.8	80.1

Table B. The effect of hypophysectomy, sham operation and pair feeding, and hypophysectomy plus growth hormone treatment on albumin synthesis. Alb. conc. = albumin concentration; P.V. = Plasma volume; I.V.P. = intravascular albumin pool; F.S.R. = Fractional synthesis rate; A.S.R. = Absolute synthesis rate.

* Unusually large plasma volumes resulted from prior sampling for catabolic rate measurements.

The effect of hypophysectomy, sham operation and pair feeding, and hypophysectomy plus growth hormone, on plasma albumin concentration (Table C), fractional and absolute catabolic rates (Tables D and E)

TABLE C PLASMA ALBUMIN CONCENTRATION (g%)

	MEAN	2 S.D.
Normal Values	2.42	0.49

	Hypophysectomy		Sham operation and pair feeding		Hypophysectomy and growth hormone	
Day	Mean	1 S.D.	Mean	1 S.D.	Mean	1 S.D.
0	2.60	0.27	2.49	0.30	2.38	0.30
1	2.29	0.39	2.57	0.10	2.11	0.33
2	2.08	0.38	2.54	0.17	1.91	0.28
3	1.98	0.46	2.60	0.23	1.78	0.35
4	*	*	2.54	0.16	1.71	0.27
5	1.91	0.29	2.50	0.37	1.86	0.28
6	*	*	2.49	0.27	2.05	0.28
7	1.82	0.32	2.45	0.19	2.08	0.31
8	*	*	2.34	0.41	1.95	0.39
9	1.74	0.35	2.34	0.37	2.00	0.35

TABLE D FRACTIONAL CATABOLIC RATE OF ALBUMIN (%I.V.P./Day)

	MEAN	2 S.D.
Normal Values	77.6	15.3

	Hypophysectomy		Sham operation and pair feeding		Hypophysectomy and growth hormone	
Day	Mean	1 S.D.	Mean	1 S.D.	Mean	1 S.D.
0	82.1	7.6	81.2	7.7	82.7	5.7
1	87.9	11.5	77.9	16.5	86.3	15.0
2	103.0	13.7	129.7	12.6	94.6	12.1
3	90.8	17.4	79.9	22.8	92.4	17.2
4	78.4	17.9	84.1	14.1	93.3	12.9
5	76.5	11.9	78.7	7.4	74.5	10.0
6	53.5	11.7	72.8	10.0	73.6	7.7
7	51.2	16.0	75.2	12.4	66.4	12.5
8	57.0	9.5	75.0	10.0	66.6	12.1
9	52.0	15.3	79.0	5.3	66.8	14.4

TABLE E ABSOLUTE CATABOLIC RATE OF ALBUMIN (mg./day)

	MEAN	2 S.D.
Normal Values	207.3	66.6

	Hypophysectomy		Sham operation and pair feeding		Hypophysectomy and growth hormone	
Day	Mean	1 S.D.	Mean	1 S.D.	Mean	1 S.D.
0	200.8	34.5	235.6	39.1	216.2	30.9
1	186.2	48.2	218.6	34.5	194.4	34.3
2	204.9	55.6	365.2	18.2	194.8	39.6
3	177.3	52.3	232.0	54.2	172.3	37.6
4	*	*	214.0	45.1	171.8	34.5
5	129.8	26.4	207.3	32.8	149.7	32.8
6	*	*	195.8	26.8	163.6	38.6
7	91.4	28.3	203.2	36.9	146.6	27.3
8	*	*	180.0	34.0	133.9	21.8
9	80.1	25.2	194.4	22.8	137.7	24.9

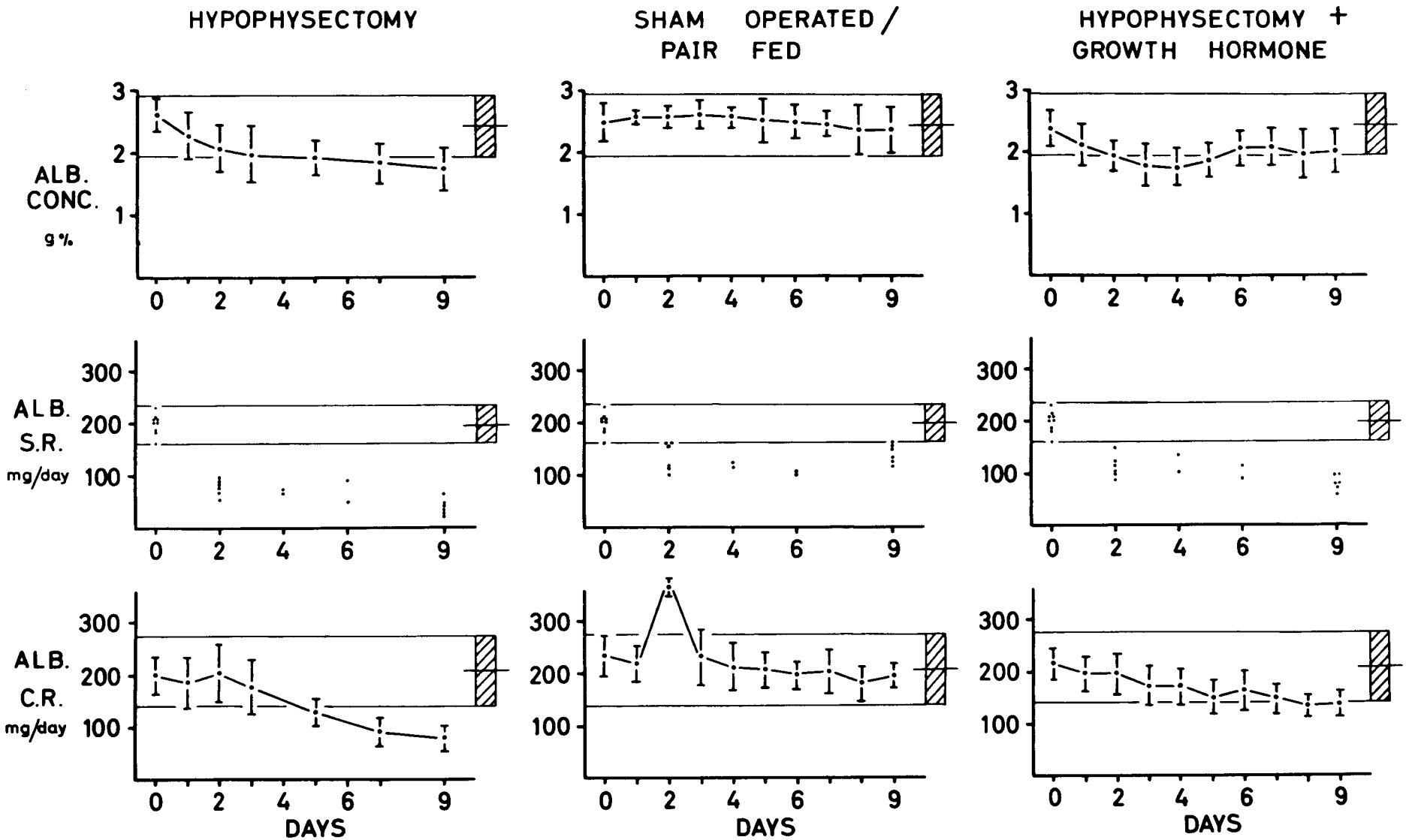

Figure 4:3 The effect of hypophysectomy, sham operation and pair feeding, and hypophysectomy plus growth hormone treatment on plasma albumin concentration and absolute synthesis and catabolic rates.

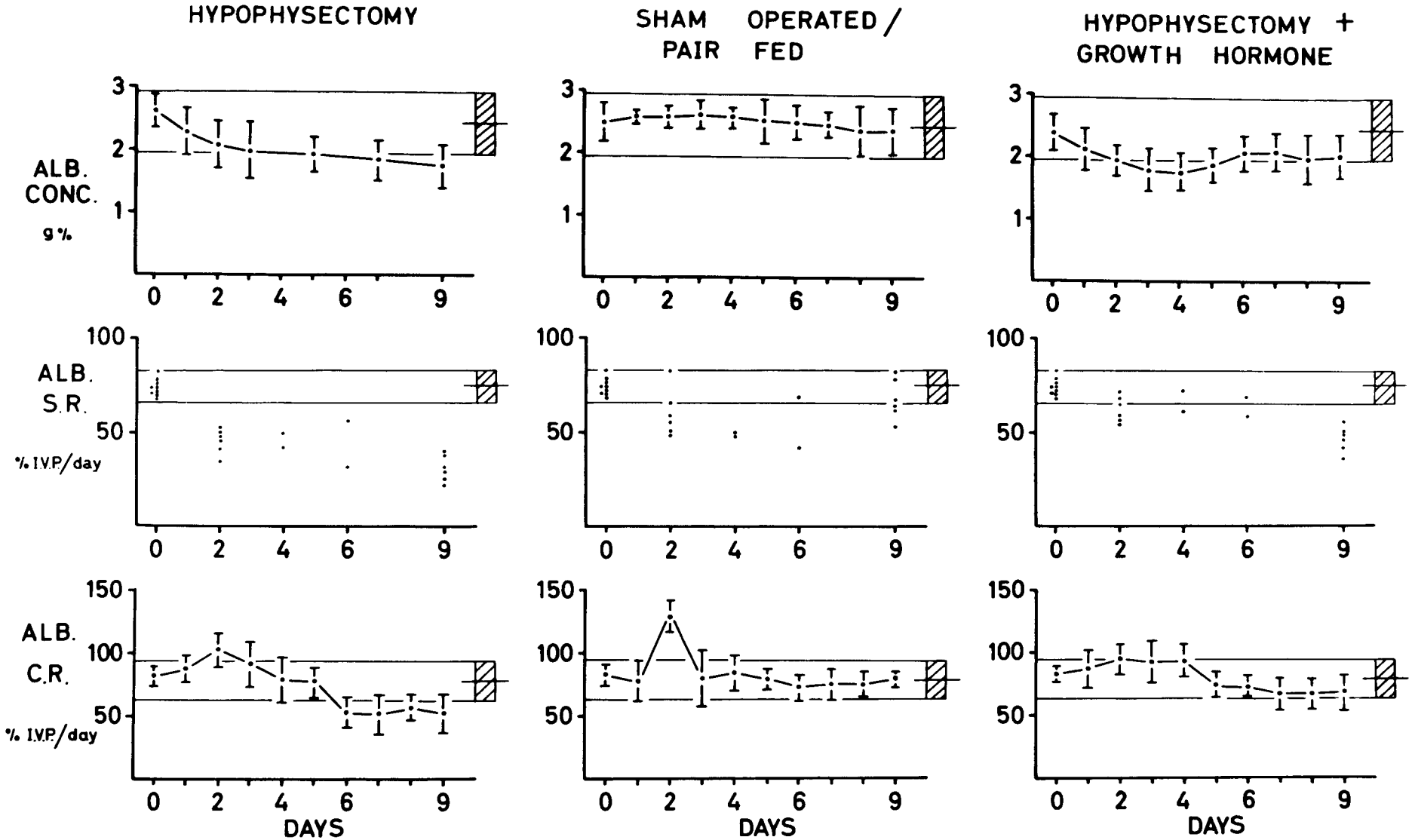

Figure 4:4 The effect of hypophysectomy, sham operation and pair feeding, and hypophysectomy plus growth hormone treatment on plasma albumin concentration and fractional synthesis and catabolic rates.

Table I. ABSOLUTE SYNTHESIS RATE COMPARISONS

	HX2	HX9	PF2	PF9	GH2
HX9	.002				
PF 2	.001	-			
PF 9	-	.001	>.409 <.469		
GH 2	.002	-	.090	-	
GH 9	-	.002	-	.001	.004

Table II. FRACTIONAL SYNTHESIS RATE COMPARISONS

	HX2	HX9	PF2	PF9	GH2
HX9	.004				
PF 2	.032	-			
PF 9	-	.001	.155		
GH 2	.008	-	.350	-	
GH 9	-	.004	-	.002	.004

Table III. ABSOLUTE CATABOLIC RATE COMPARISONS

	HX2	HX9	PF2	PF9	GH2	
HX9	0 7/14					
PF 2	0 1/5	-				
PF 9	-	0 1/5	P=.004			
GH 2	43 7/14	-	0 1/5	-		
GH 9	-	3 7/14	-	1 1/5	8 7/14	GH 9

Table IV. FRACTIONAL CATABOLIC RATE COMPARISONS

	HX2	HX9	PF2	PF9	GH2	
HX9	0 7/14					
PF 2	3 1/5	-				
PF 9	-	2 1/5	P=.004			
GH 2	29 7/14	-	0 1/5	-		
GH 9	-	17 7/14	-	12 1/5	4 7/14	GH 9

The significance of the difference between the absolute and fractional synthesis (Tables I & II) and catabolic (Tables III & IV) rates on days 2 and 9 in the three groups of rats are presented above. By reading the value at the intersection of the appropriate horizontal and vertical columns, the significance of the difference between any two groups may be determined. HX = Hypophysectomy, PF = sham operation and pair feeding, GH = Hypophysectomy plus growth hormone. The figure 2 or 9 after these abbreviations refers to the second or ninth post-operative day. Tables I and II provide exact values for P, Tables III and IV provide critical values of 'U' at .001 and .01 significance levels.

Table V. COMPARISON BETWEEN EXPERIMENTAL SYNTHESIS RATE VALUES AND NORMAL VALUES ON DAY 2 AND 9

	HX2	HX9	PF2	PF 9	GH 2	GH 9
Absolute SR	$0\ \frac{2}{7}$	$0\ \frac{2}{7}$	$0\ \frac{2}{7}$	$0\ \frac{2}{7}$	$0\ \frac{2}{7}$	$0\ \frac{2}{7}$
Fractional SR	$0\ \frac{2}{7}$	$0\ \frac{2}{7}$	$8.5\ \frac{2}{7}$	$16\ \frac{2}{7}$	$3\ \frac{2}{7}$	$0\ \frac{2}{7}$

The significance of the difference between the experimental absolute or fractional synthesis rates and normal rates is obtained by choosing the appropriate horizontal and vertical column; e.g. the pair fed fractional synthesis rate on day 2 is not significantly different from normal at either the .001 or .01 levels $(8.5\ \frac{2}{7})$. Critical values of 'U' are provided.

Table VI. COMPARISON BETWEEN SYNTHESIS AND CATABOLIC RATES ON DAYS 2 AND 9.

	HX2	HX9	PF 2	PF 9	GH 2	GH 9
Absolute	$0\ \frac{2}{7}$	$4\ \frac{2}{7}$	P = .002	P = .002	$0\ \frac{2}{7}$	$2\ \frac{2}{7}$
Fractional	$0\ \frac{2}{7}$	$3\ \frac{2}{7}$	P = .002	P = .089	$0\ \frac{2}{7}$	$2\ \frac{2}{7}$

The significance of the difference between the synthesis and catabolic rates in any one group of experimental rats on day 2 or 9 may be determined by choosing the appropriate horizontal and vertical columns; e.g. in the pair fed rats on day 9 the fractional synthesis rate is not significantly different from the fractional catabolic rate since P = .089. Where possible exact values of P are provided, otherwise critical values of 'U' are presented.

EFFECT OF HYPOPHYSECTOMY

A. Synthesis rate

(i) Compared with normal values.

After Hypophysectomy both absolute and fractional synthesis rates fell sharply and forty eight hours post-operatively were highly significantly less than normal (Table V). The values on days 4 and 6 were of the same magnitude but the day 9 values displayed a further slight fall with the result that not only were they highly significantly less than normal, but also significantly less than the day 2 values. (Tables I and II).

B. Catabolic rates

(i) Compared with normal values.

The absolute catabolic rate remained within normal limits for approximately four days but then fell and was highly significantly less than normal on the fifth day post-operatively ($5\frac{19}{31}$) The fall was initially rapid, but became less pronounced in the latter part of the study period.

The fractional catabolic rate displayed a highly significant post-operative rise above normal values on day 2 ($4\frac{19}{31}$). Thereafter the catabolic returned to normal levels but continued to fall and from day 6 onwards was highly significantly less than normal. ($10.5\frac{19}{31}$)

(ii) Synthesis and catabolic rates compared.

Absolute and fractional rates of catabolism were at all times higher than the corresponding synthesis rates. On day 2 catabolic rates were highly significantly greater, but because of the reduction in catabolism, differences on day 9 were less marked although still significant (Table VI).

C. Albumin Concentration

The plasma albumin concentration displayed a rapid fall during the first three days of the study period. Thereafter the fall was less steep, the mean

concentration / .....

concentration falling below the second standard deviation of the normal values on day 7.

EFFECT OF SHAM OPERATION AND PAIR FEEDING

A. Synthesis Rates

(i) Compared with normal values.

Sham operation and two days of pair feeding resulted in a fall in the absolute synthesis rate to values that were highly significantly less than normal, (Table V). The absolute synthesis rate continued to fall on days 4 and 6 but although the values rose on day 9, they were still highly significantly less than the normal (Table V). The values on day 2 and 9 were not significantly different from each other (Table I).

The fractional synthesis rate though falling, was only probably significantly less than normal on day 2 (U (obs) = 8.5, U at 0.5 level = 12). The values on day 4 were of the same order, but the two values on day 6 were widely separated. The fractional rates on day 9 showed a continuing rise and were not significantly less than normal values (Table V) nor significatnly different from the day 2 values (Table II).

(ii) Compared with the synthesis rate in hypophysectomised rats.

The absolute synthesis rate in the pair fed groups on days 2 and 9 was highly significantly greater than that of the hypophysectomised group (Table I). The fractional synthesis rates of the pair fed group on day 2 was only probably significantly greater than the hypophysectomised group but on day 9 was highly significantly greater (Table II).

B. Catabolic Rate

(i) Compared with normal values.

The response of both the absolute and fractional catabolic rates to

sham operation / .....

sham operation and pair feeding was similar. The post-operative rise became highly significantly greater than normal on day 2 ($0-\frac{5}{12}$), after which there was a return to the normal range, within which the values remained for the duration of the study period.

(ii) Synthesis and catabolic rates compared.

The absolute and fractional catabolic rates were at all times greater than the corresponding synthesis rates. Absolute rates were significantly different on days 2 and 9 (Table VI). Fractional rates differed significantly on day 2, but there was no significant difference on day 9 (Table VI).

(iii) Compared with the catabolic rate in hypophysectomised rats.

The absolute catabolic rate in the pair fed group was highly significantly greater than that of the hypophysectomised group (Table III). Differences in the fractional catabolic rate were less marked but still significant (Table IV).

C. Albumin concentration

The plasma albumin concentration remained within normal limits throughout the nine day study period.

EFFECT OF HYPOPHYSECTOMY PLUS GROWTH HORMONE TREATMENT

A. Synthesis Rate

(i) Compared with normal values.

The absolute synthesis rate after hypophysectomy and two days of growth hormone treatment was highly significantly less than normal (Table V) and furthermore fell progressively during the remainder of the study period.

The fractional synthesis rate behaved in an essentially similar fashion. On day 2 this was significantly less than normal and because there was a continued fall, the difference on day 9 became highly significant (Table V).

Absolute and /.....

Absolute and fractional synthesis rates on day 9 were significantly less than those on day 2 (Tables I and II).

(ii) Compared with the synthesis rate in the untreated hypophysectomised rats.

On days 2 and 9 the absolute and fractional synthesis rates of the growth hormone treated rats were significantly greater than those of the untreated hypophysectomised rats (Table I and II).

(iii) Compared with the sham operated pair fed rats.

On day 2 the absolute and fractional synthesis rates in these two groups were not significantly different (Tables I and II). However, on day 9 the absolute rates in the treated rats were highly significantly less than those of the pair fed rats. Differences in fractional rates were less marked but still significant (Tables I and II).

B. Catabolic Rate

(i) Compared with normal values.

The absolute catabolic rate fell slowly throughout the study period, becoming significantly less than normal from day 7 onwards, $(8 - \frac{19}{31})$. There was no early post-operative rise.

The fractional catabolic rate showed a post-operative rise to a value that was highly significantly greater than normal on day 2, $(17 - \frac{19}{31})$. The catabolic rate then fell, and from day 8 was significantly less than normal, $(29 - \frac{19}{31})$.

(ii) Synthesis and catabolic rates compared.

At all times the absolute and fractional catabolic rates were highly significantly greater than the corresponding synthesis rates (Table VI).

(iii) Compared / .....

(iii) Compared with the catabolic rate in the untreated hypophysectomised rats.

The absolute and fractional catabolic rates in the treated and untreated hypophysectomised rats were not significantly different on day 2 (Tables III and IV). On day 9 however, the absolute catabolic rate was highly significantly greater in the treated rats but there was no significant difference between the fractional catabolic rates on this day (Tables III and IV).

(iv) Compared with the catabolic rate in the sham operated pair fed rats.

Absolute and fractional rates in the growth hormone treated group were highly significantly less than those of the pair fed group on day 2. The same was true of the absolute rates on day 9, but there was no significant difference between the fractional rates on this day.

C. Albumin Concentration

The albumin concentration fell sharply for the first four days after which there was a rise so that for the last four days of the study period, they lay within the lower limit of normal.

CHAPTER FIVE

DISCUSSION

It has long been recognised [13, 35, 40, 41, 43, 51] that the hypophysectomised rat voluntarily consumes less food than normal rats. Whilst the cause of this anorexia has not been determined with certainty, if inference from clinical experience with hypopituitary patients is permitted, it seems most likely that glucocorticoid deficiency is the underlying cause since this symptom is consistently abolished by cortisone treatment. Additional support for this contention comes from limited experiments conducted but not included in this thesis, in which corticosterone was administered in physiological doses to hypophysectomised rats and resulted in a marked improvement in the food intake. It would appear that growth hormone deficiency is not the responsible factor since no increase in food intake was observed when hypophysectomised rats were treated with growth hormone, [148] a finding which has been confirmed in these studies. Inadequate protein consumption may result in significant alterations in albumin metabolism and the interpretation of the data obtained in the untreated and growth hormone treated hypophysectomised rats must take into account the influence of the post hypophysectomy anorexia.

Over the past decade numerous studies have focussed attention on the effects of altered dietary intake on albumin metabolism, from which much of our present day understanding of albumin homeostasis has been derived. Since many of the observations made in the experiments in the treated and untreated hypophysectomised rats can be explained in terms of principles established in these nutritional studies, a discussion of the results of the pair feeding experiments will be presented first, along with a brief review of the relevant literature.

1. PROTEIN DEPRIVATION AND ALBUMIN METABOLISM

Hypoalbuminaemia is a characteristic feature of kwashiokor [149 - 154] and is a reflection of the depletion of the total body albumin mass. Brock [155] and Waterlow [156] were agreed that significant hypoalbuminaemia occurred as a late event, and that considerable depletion of body protein could occur before the plasma

albumin level / .....

albumin level was significantly reduced.

When faced with an inadequate supply of dietary protein, losses of albumin occur from both the intra and extravascular compartments. Although methods for measuring the extravascular albumin mass are, in the strictest sense, only valid under steady state conditions, it has been demonstrated repeatedly (101, 157 - 159, 161 - 163) that when hypoalbuminaemia is produced by either dietary protein restriction or by plasmaphoresis, depletion of the extravascular mass exceeds that of the intravascular mass. Evidence was also presented in these studies to show that albumin had been transferred from the extravascular to intravascular compartments, and it was proposed that this transfer represented an adaptive response on the part of the body to maintain the intravascular albumin pool constant, albeit at the expense of the extravascular pool. Though initially such transfer might succeed in its purpose, ultimately in the face of continued insult, the intravascular albumin pool would also become depleted. It is therefore easy to conceive of a situation in which depletion of total body albumin might occur to some degree before a reduction in plasma albumin concentration ocurred, and such a situation was reproduced by Hoffenberg et al (161) by exposing adult subjects to a low protein diet.

Reduced rates of albumin catabolism are found in children suffering from kwashiorkor, (156, 158, 163 - 165) as well as in experimental subjects (161) or animals (127, 160, 161, 166, 167) exposed to diets low in protein content, and this reduction in catabolic rate appears to be related to the state of depletion of the subject rather than to the protein intake at the time of measurement. For example, Hoffenberg et al (161) found that catabolic rates in hypoalbuminaemic subjects were low despite the provision of a normal dietary intake. Kirsch et al (127) showed that when rats were acutely deprived of dietary protein the catabolic rate only fell approximately one week after the protein free diet was started. Conversely when these protein depleted rats were once again allowed to eat a diet containing normal levels of protein, catabolism did not increase immediately but some three or four days later. Comparable observations were made by James and Hay (163) who conducted similar experiments in infants suffering from kwashiorkor.

The evidence to suggest / .....

The evidence to suggest that it is the size of the intravascular pool of albumin (reflecting as it does the state of depletion) that dictates the rate of catabolism, is compelling. Analbuminaemic subjects have abnormally low rates of catabolism [169, 170] which can be restored to normal if the intravascular pool is expanded by albumin infusion. [171] Above normal rates of catabolism were obtained after albumin infusions in mice, [168] rabbits [172] and humans. [173] Conversely depletion of the intravascular pool by plasmaphoresis [101, 161] caused a reduction in albumin catabolism, despite the provision of a normal diet.

The elucidation of the behaviour of albumin synthesis in dietary depletion has been hindered by the absence of valid methods for measuring this process under nonsteady conditions, as was discussed in general terms in Chapter 2. "Synthesis plus transfer" rates were found to be low in rats [166] and rabbits and humans [161] on low protein diets. When protein was again made available, albumin levels rose rapidly to normal, from which it was inferred [158, 165] that initiation of cure was accompanied by a considerable increase in albumin synthesis.

Recently changes in albumin synthesis have been measured directly. Hoffenberg, [142] Kirsch et al [127] and Rothschild et al [124] have used the ^{14}C carbonate method and James and Hay [163] have used ^{131}I labelled albumin and a computer technique suitable for nonsteady conditions. Unlike albumin catabolism, withdrawal of protein from the diet leads to a prompt reduction in albumin synthesis and repletion results in a rapid return to normal or even above normal levels. This behaviour strongly suggests that albumin synthesis is intimately related to the availability of substrate amino acids.

The overall responses of albumin metabolism to protein deprivation and repletion may therefore be summarised as follows:

> Withdrawal of dietary protein rapidly results in a reduction in albumin synthesis, and since catabolism within the intravascular compartment continues to proceed at normal rates, the intravascular albumin pool starts to contract. Increased transfer of albumin from the extravascular compartment might protect the intravascular compartment from depletion to an extent but, when the intravascular

albumin mass shrinks to some critical level, catabolism becomes reduced as an additional protective measure. When an adequate protein intake is again made available synthesis rapidly returns to normal levels and the intravascular albumin pool is gradually expanded until such time as the compensatory reduction in catabolic rate is no longer required.

Discussion of the results of the pair feeding experiments.

(i) SYNTHESIS RATE

As was anticipated the pair feeding experiments resulted in changes in albumin metabolism. During the first two days of the experimental period the food intake was severely restricted, the rats being provided with an average of only 3 g. of food, which virtually amounted to a total fast. The mean absolute synthesis rate on day 2 was 32% less than the mean normal value, and this fall represented a highly significant reduction. Though the mean fractional synthesis rate was 19% less than the mean normal, this represented a change that was at best only probably significant.

Kirsch et al [127] showed that the absolute synthesis rate became significantly reduced after approximately six days of protein deprivation. Those findings and the present are not strictly comparable however since in the present study the rats were fed a normal diet in reduced quantities, whereas in the former study the protein free diet was made isocaloric on a weight basis with a normal diet, and ad libitum feeding was permitted. Under such circumstances one might anticipate a protein sparing action of carbohydrate.

Rothschild et al [124] have studied the effect on albumin synthesis of an eighteen to thirty-six hour fast in rabbits. After this time interval the absolute synthesis rate was found to be reduced by 33%. Fractional synthesis rates were not provided but since body weight, plasma volume and plasma albumin concentration were virtually unchanged before and after the fast, the reduction in synthesis rate must have been due almost entirely to a lowered fractional rate. In the present studies absolute synthesis was reduced by an almost identical amount as that observed by Rothschild but the reduction in fractional rate was less marked. The

greater fall / .....

The greater fall in absolute than fractional synthesis rates when compared with normal values was due to the presence of a reduced intravascular albumin pool in the experimental rats. Contraction of the pool size resulted from the combined effects of the sham operation and food restriction causing marked body weight loss and a smaller plasma volume. Thus, four of the six batches studied had plasma volumes that fell outside the range of the normal rats, but the albumin concentration remained within the normal range in all.

The present study, as well as that of Rothschild et al, [124] not only confirms the well established finding of reduced albumin synthesis when dietary protein is inadequate, but demonstrates the rapidity with which this reduction can occur. Whilst the basic defect is clearly a lack of substrate amino acids in the diet, Rothschild et al [124] also showed that some change, as evidenced by reduced amino acid incorporating ability, occured in the microsomes of liver parenchymal cells of the fasted rabbits. Korner [174] however in an earlier study was unable to show this effect in the rat liver cell free system after an eighteen hour fast.

After day 2 the food intake increased progressively, though still remaining well below the normal range. Synthesis rates remained low until day 9, by which time sufficient food was being provided to maintain normal fractional synthesis rates in three of the six batches studied. As a group the fractional synthesis rates on day 9 were not significantly different from normal though the absolute synthesis rates were. This difference in significance is again explained by the smaller size of the experimental animals compared to the normal controls.

Kirsch et al [127] and James and Hay [163] have clearly demonstrated that normal synthesis rates can be re-established as rapidly as twenty-four hours after the provision of adequate amounts of dietary protein, and a similar rapid response has been shown here when the food intake was increased in the latter half of the study period. There is clearly individual variation in the amount of food needed to support normal synthesis rates, since only three of the six batches consuming larger quantities of food on day 9 had normal fractional synthesis rates, whilst one of the two batches on day 6 had a normal rate despite a smaller food intake.

(ii) Catabolic Rate/.....

(ii) CATABOLIC RATE

Apart from a highly significant rise on day 2, absolute and fractional catabolic rates remained within normal limits throughout the study period. The relation between the introvascular pool size and the albumin catabolic rate was discussed on page 60 . In terms of this relationship the presence of the normal catabolic rate (apart from the post-operative rise) indicates that the intravascular pool had not been compromised sufficiently (as reflected by the normal albumin concentration) by reduced synthesis to require the compensatory reduction in catabolism.

The nature of the post-operative rise in catabolism.

Tissue injury, for example burns, fractures and surgical operation results in a number of biochemical responses. Negative nitrogen balance and hypoalbuminaemia are two such responses. Increased rates of albumin catabolism have been demonstrated after fractures [175] and burns, [175, 176] and post-operatively.[176 - 178] Though increased rates of catabolism have been convincingly demonstrated, exudation of albumin into wounds (and in particular burns) may contribute to a variable extent to the increased rate of elimination of albumin from the intravascular pool. [176, 179, 180]

Similarities between the biochemical responses post-operatively and after administration of glucocorticoids initially prompted the measurement of glucocorticoid activity in patients suffering tissue injury or undergoing surgery. Raised urine and plasma 17 hydroxycorticosteroids have been demonstrated in a wide variety of conditions, [182 - 184, 186 - 189] and the rise appears to correlate with the "stress" to which the patient is exposed. In uncomplicated surgery raised 17 hydroxycorticosteroid levels exist for twelve to twenty-four hours [184, 187, 188] whilst in burns the elevation may persist for a week or longer. [185] In view of the ability of glucocorticoids to stimulate albumin catabolism in experimental [132, 190] or naturally [191, 192] occuring situations, a satisfactory explanation to account for increased albumin catabolism would be on the basis of raised glucocorticoid activity. Cuthbertson [181] has reviewed the evidence and has concluded that increased adrenocortical secretion is clearly involved but that this is not the sole mediator of the

metabolic / .....

metabolic response to injury.

Glucocorticoid levels were not measured in the sham operated pair fed rats. If it is assumed that the raised levels of albumin catabolism coincide with elevated glucocorticoid levels, then a twenty-four to forty-eight hour post-operative adrenocortical response would have occured, and this would agree with the magnitude of the response shown by other workers after uncomplicated surgery. Retention of radioiodide in the body water which could have resulted in spuriously high values for catabolism, was excluded by measuring T.C.A. precipitable activity in the plasma. No excess unbound activity was demonstrated in the plasma, and neither was there any wound infection or oedema into which labelled albumin may have been exuded, so that it may be accepted that the high post-operative values were due to a true increase in catabolism. That this increase was mediated at least in part by raised glucocorticoid activity is unproven, but appears to be likely in view of the much smaller post-operative response exhibited by the hypophysectomised rats in which the pituitary-adrenal axis was disrupted and continued adrenocorticoid function prevented.

II. HYPOPHYSECTOMY AND ALBUMIN METABOLISM

The earlier experimental work which was reviewed in Chapter 1 demonstrated conclusively that significant reductions in plasma albumin levels ocurred after hypophysectomy, and furthermore showed that a true reduction in intravascular albumin mass ocurred. These results whilst consistent and convincing were of limited value since the underlying changes in synthesis or catabolism could not be defined. Ulrich and coworkers [51] were the first to provide such information. A tracer dose of ^{35}S labelled albumin was administered to albino rats two weeks after hypophysectomy and the plasma albumin specific activity measured over the next seven days. Using a kinetic model based essentially on the slope of the plasma specific curve, these workers deduced that after hypophysectomy a fall in synthesis rate occured initially followed by a fall in catabolism, and that the albumin "replacement rate" was approximately half that of normal rats. Because of the assumptions introduced by the authors when considering their data, this study offers some difficulty in interpretation. In the first instance it was assumed that synthesis and catabolism comprised a single

process and only / .....

process and only a net change in "synthesis-catabolism" could be determined. Secondly the authors assumed steady state conditions to have prevailed during the experiment, which it was conceded by them, in view of the rapid weight loss evidenced by the animals could not have obtained. From the general considerations presented in Chapter 2 it will be recalled that turnover data derived from the slope of the plasma specific activity curve alone fails to take into account specific activity differences between intra and extravascular compartments. The kinetic model employed in this study is therefore clearly inappropriate and the difficulty in interpreting this data is compounded by the use of an isotopic label that is subject to a degree of re-utilisation.

The only other study where the kinetic behaviour of albumin after hyphysectomy has been investigated is the clinical study of Jeejeebhoy et al, [52] who used radioiodinated albumin to measure albumin catabolism and "synthesis plus transfer" in hypopituitary subjects, and found that three of four cases studied had values for these processes below the normal control range. This study whilst providing representative values for catabolism, only provides an approximate value for synthesis. As was emphasised in Chapter 2, the study of iodinated albumin preparations can never supply direct information about rates of albumin synthesis. It should also be mentioned that all of the cases studied were on some form of hormonal substitution therapy (exclusive of growth hormone) and the results obtained may not truly reflect the state in the untreated hypopituitary subject.

The results of the experiments conducted in this thesis provide the first information regarding the effect of hypophysectomy on albumin synthesis and catabolism when these processes are measured directly and independently of each other.

Discussion of the results of the hypophysectomy experiments:

(i) SYNTHESIS RATE

Compared with normal values, hypophysectomy resulted in an early and striking reduction in both absolute and fractional synthesis rates, and a further significant reduction occurred at the end of the study period: The reduction in synthesis rate may have resulted from the single or combined effects of (a) a deficiency of one or more of the pituitary hormones and (b) the reduction in food intake.

(a) There is a / .....

(a) There is a paucity of data relating to the role of pituitary hormones in albumin synthesis. That relating to growth hormone has been discussed in Chapter 1 and will be returned to in greater detail later in this chapter. Synthetic analogues of androgens have not been shown to have any effect on albumin metabolism. (131, 196) Adrenocortical (132, 190 - 192) and thyroid (130, 193 - 195) hormones have been shown to increase albumin catabolism 'in vivo', and since the total amount of albumin estimated to have been catabolised was greater than the amount by which the total body albumin mass was reduced, it was concluded that albumin synthesis had increased to compensate for the increased catabolism. That synthesis was stimulated directly was not ascertained, and the ability of glucocorticoids and thyroid hormones to stimulate albumin synthesis remains to be demonstrated by direct measurement. To date no such measurements have been reported. Further discussion of the possible role of pituitary hormones in albumin synthesis will be undertaken when the effects of growth hormone treatment are presented.

(b) On day 2 there was no significant difference between the fractional synthesis rates of the hypophysectomised rats and that of the pair fed rats so that the immediate effect after hypophysectomy was no greater than could be accounted for by a reduced food intake. However, at the end of the study period clearcut differences manifested. Whereas the sham operated rats when fed larger quantities of food immediately responded by restoring albumin synthesis to normal rates, the hypophysectomised rats displayed a further significant fall and the differences in synthesis rate between the two groups of rats on day 9 became highly significant. On day 9 therefore the reduction in synthesis rate was greater than would be expected on the basis of a reduced food intake, and pituitary insufficiency was clearly contributory. The probable nature of this insufficiency will be discussed later in this chapter.

Prior to embarking on this project it was thought that after hypophysectomy the synthesis rate would fall more rapidly than after sham operation, for after the latter the pituitary anabolic hormones would still be present - that was of course at that time assuming that the pituitary gland did indeed participate in albumin synthesis. This has not been borne out by the results of the experiments since the fall in synthesis rate in

the first two days / .....

the first two days after hypophysectomy was as rapid and as great as that after fasting, so that the presence of the pituitary gland confers no particular advantage on the individual in the face of protein depletion. What has become apparent however is that the pituitary gland is important for the restoration of normal rates of synthesis during the process of repletion. In the face of a larger food intake the sham operated but not the hypophysectomised rats were able to synthesise albumin at normal rates. It seems reasonable to suggest therefore that the rapid return to normal levels of albumin synthesis observed by Kirsch et al [127] and James and Hay [163] after protein repletion, is a response for which an intact and functioning pituitary gland is required.

In view of the continued fall in synthesis rate despite the larger food intake, one must ask if the hypophysectomised rat is able to utilise any of the food it eats. The issue is largely speculative but it should be noted that Levin [37] was unable to cause growth or to establish positive nitrogen balance when hypophysectomised rats were force fed normal quantities of food.

(ii) CATABOLIC RATE

The post-operative rise in catabolism on day 2 was evident only in the fractional rate, since the rapidly falling albumin concentration obscured this response when the catabolic rate was expressed in absolute terms. The post-operative response in the hypophysectomised rats was significantly less than in the sham operated rats, confirming that the pituitary gland plays an integral part in the post-operative rise in albumin catabolism. Although significantly less than the pair fed rats, the fractional catabolic rate on day 2 in the hypophysectomised rats was still significantly greater than normal. This more moderate response presumably reflects the effects of only ten to fifteen minutes of anaesthesia and surgery prior to removal of the pituitary gland whilst continued pituitary function remained possible in the sham operated rats.

For the fall in the catabolic rate at the end of the study period two possible explanations present themselves:-

(1) Absence of / .....

(1) Absence of pituitary hormones.

Adrenocortical [132, 190 - 192] and thyroid [130, 193 - 195] hormones have been shown to increase albumin catabolism. It becomes possible to argue therefore that catabolism would be decreased once these hormones disappeared after hypophysectomy. For this reason corticosterone levels were measured in the plasma after hypophysectomy. Complete disappearance had occurred within forty-eight hours, whereas the fall in catabolism only occurred three to four days later. If plasma corticosterone levels may be taken as correlating with their biological effect in general (and from clinical experience this assumption appears to be largely justified) then the reduction in catabolism cannot be ascribed to the disappearance of glucocorticoids.

Similar measurements of thyroid hormones were not undertaken. Clinical experience has shown that the effects of circulating thyroid hormones take some days to wear off. The possibility that the reduction in catabolism was due to a reduction in thyroid hormone action some days after hypophysectomy, cannot therefore be excluded.

(2) Reduction in albumin intravascular pool.

Marked reductions in the intravascular pool size occurred as a result of the profoundly reduced synthesis rate, and is reflected by the lowered plasma albumin concentration. The compensatory reduction in catabolism would occur in response to a decreased intravascular pool irrespective of the pathogenesis. Since however the decreased diet was not sufficient to reduce catabolism in the sham operated rats, reduction in catabolism in the hypophysectomised rats may be ascribed to hypopituitarism causing decreased synthesis and consequently a reduced intravascular pool.

III GROWTH HORMONE AND ALBUMIN METABOLISM

It is clear from the review of the literature presented in Chapter 1 that there is considerable conflict of opinion as to the role played by growth hormone in regulating albumin metabolism. Administration of growth hormone to intact animals had little or no effect on the albumin concentration [53 - 55] but caused an increase in

the intravascular / .....

the intravascular pool size. [53] In hypophysectomised animals growth hormone had little [45] or no effect [49, 50] on restoring lowered albumin levels to normal. Although in one study [50] growth hormone was able to restore the intravascular pool to its normal size, the fact that the growth hormone preparation used was heavily contaminated with other pituitary hormones must cast some doubt on the validity of the results.

As far as the dynamic effects of growth hormone on albumin metabolism are concerned, the picture is as confusing. The only two studies which have endeavoured to clarify this aspect in hypophysectomised subjects are those of Ulrich et al [51] and Jeejeebhoy et al. [52] These two studies were evaluated when considering the results these workers obtained after hypophysectomy, and the same criticisms levelled there are also applicable to the observations made after growth hormone replacement therapy. It was shown by Ulrich et al that treatment with growth hormone resulted in a steeper plasma albumin specific activity curve and an increase in the body albumin pool. The albumin "replacement rate" was nearly three times that of the untreated hypophysectomised rats, and these findings were interpreted to mean that growth hormone caused an increase in albumin synthesis. On the other hand Jeejeebhoy et al found that the "synthesis plus transfer" and catabolic rates already low in hypopituitary humans were further decreased when growth hormone was administered, and it was suggested by these workers that growth hormone achieved its effect by decreasing albumin catabolism rather than by increasing its synthesis. These findings raise the important question as to whether growth hormone is anabolic or anti-catabolic in respect of albumin metabolism and attention will be paid to this point on page 74.

Experimentation with growth hormone in intact subjects has also led to conflicting results. Gabuzda et al [57] attempted the separate measurement of albumin synthesis and catabolism after the injection of ^{131}I labelled albumin in four humans with clinical and biochemical evidence of hepatic cirrhosis. The difficulties of this form of measurement have already been outlined and interpretation is furthermore made difficult by the choice of subjects with abnormal liver function. From the data obtained the authors concluded, as had Jeejeebhoy, that growth hormone

reduced albumin / .....

reduced albumin catabolism but had no effect on synthesis. Gross et al [56] believed that growth hormone had no effect on either synthesis or catabolism but postulated that under the conditions of their experiment, growth hormone raised the plasma albumin concentration by altering capillary permeability in such a way as to reduce the outflow of albumin from intravascular to extravascular compartments. The only study where albumin synthesis has been measured directly by the ^{14}C carbonate method, is an 'in vitro' study undertaken by Sellers et al [59] using a perfused rat liver. In this way a 33% increase in synthesis rate was demonstrated if the donor rats were pretreated with growth hormone.

Discussion of the growth hormone replacement experiments.

(i) SYNTHESIS RATE.

The present experiments provide the first demonstration of the ability of growth hormone to stimulate albumin synthesis 'in vivo' when measured directly. Both at the beginning and the end of the study period absolute and fractional synthesis rates in the growth hormone treated rats were significantly higher than those of the untreated rats, the latter proven by immunoassay to be growth hormone deficient. However, despite this ability to stimulate albumin synthesis the values obtained were still significantly less than normal and a consideration of the reasons why normal values were not obtained is warranted.

In the first instance it was observed that growth hormone had no significant effect on the post hypophysectomy anorexia and as a result the food intake was still considerably less than normal. Under these circumstances therefore the best effect growth hormone could be expected to achieve, would be to increase the synthesis rate to the level of the pair fed rats. On day 2 this effect was achieved and the values obtained in the two groups were not significantly different. One may conclude therefore that on day 2 the decreased food intake alone was preventing the restoration of normal synthesis rate levels. On day 9 however the picture was different. Despite the progressive increase in food intake synthesis rates fell throughout the study period. In the face of this increased food intake the synthesis rate in the sham operated rats rose, but fell in the growth hormone treated rats, and the difference between the two groups became significant. The fall in

synthesis rate / .....

synthesis rate in the face of both an increase in food intake and the wherewithall to synthesise albumin (viz. growth hormone) clearly implicates the participation of other factors in the regulation of the synthetic process. The possible participation of other pituitary hormones requires to be considered.

(a) Glucocorticoids:

The albumin catabolic effect of glucocorticoids is well recognised, but there is also evidence to suggest that these hormones may play a part in regulating albumin synthesis. The administration of glucocorticoids stimulates the catabolism of extra-hepatic (carcass) protein [197 - 199] and induces a state of negative nitrogen balance in the body as a whole. The loss of protein does not affect the entire body for under these conditions the viscera (liver and gut) retain or increase their weight, protein and RNA content. [197 - 200] These findings have been interpreted [199, 201, 202] to mean that glucocorticoids cause a flow of amino acids from carcass to liver. Though most of the amino acids arriving at the liver are destined for gluconeogenesis, Korner [202] believes that with the flooding of the liver with amino acids any surplus will be used for protein synthesis. This belief conceives of glucocorticoids playing a passive role in protein synthesis by providing a supply of amino acids to the liver from which protein will be synthesised under the influence of the protein anabolic hormones. Growth hormone on the other hand appears to have an opposite effect by favouring the deposition of protein in muscle, at the expense of liver protein. [204 - 206]

A more specific stimulus to protein synthesis appears likely in view of the observation of Korner [202] that cortisol enhances the ability of liver microsomes to incorporate amino acids into protein 'in vitro', and Bancroft et al [203] have recently shown that hydrocortisone caused a fourfold increase in the rate of albumin production in an albumin producing hepatic cell culture system.

The above evidence makes it possible to offer some explanation for the incomplete restoration of albumin synthesis rates in the hypophysectomised rats receiving only growth hormone treatment. The absence of glucocorticoids deprives the liver of a direct stimulus to albumin synthesis, as well as depriving it of an endogenous supply of substrate amino acids from carcass protein. Furthermore in the absence of the counter-

acting influence / .....

acting influence of glucocorticoids, the growth hormone given to the hypophysectomised rat would tend to cause a predominant shift of endogenous and dietary amino acids away from liver to muscle, thus further depriving the liver of amino acids for albumin synthesis.

(b) Thyroid hormones:

What has been said for glucocorticoids appears to be applicable to thyroid hormones. With thyroid treatment liver weight, protein and RNA content gains at the expense of that of carcass muscle, [206, 207] again suggesting that the liver is responding to an increased flow of amino acids coming from the breakdown of carcass protein. However many investigators [208 - 212] have demonstrated a stimulation by thyroid hormones of some of the intracellular events usually associated with protein synthesis (increased amino acid uptake, shift of polysome profile to heavier aggregates, increased RNA synthesis), and the appearance of albumin in the plasma of the tadpole during metamorphosis may be induced by treatment with thyroid hormones, [213] so that it appears that the response of liver protein metabolism after thyroid hormone treatment is not explicable only in terms of a passive response to an increased amino acid supply from the carcass. These observations however do no more than reflect liver protein synthesis as a whole. Whether albumin production is increased remains as yet undetermined.

In their own right thyroid hormones are essential for normal growth and development. It is also well known that normal thyroid function is necessary for the optimal expression of growth hormone. In the growth hormone treated rats therefore incomplete growth hormone effect might also have been due to the absence of the augmenting action of the thyroid hormones.

Finally, the diminishing effect of growth hormone treatment on albumin synthesis may have been due to the development of antibodies to bovine growth hormone, although there is little likelihood of this having occurred during the short space of nine days. There is little doubt that the growth hormone preparation used was biologically active since the magnitude of the post hypophysectomy weight loss was less, and blood urea levels were considerably lower than in hypophysectomised rats.

Furthermore / .....

Furthermore, this same preparation was shown by the National Institutes of Health to cause the resumption of growth in young hypophysectomised rats.

(ii) CATABOLIC RATE.

As in the untreated hypophysectomised rats the post operative rise in catabolic rate was evident only in the fractional rate, being obscured by the falling albumin concentration when expressed in absolute terms. This response was significantly less than that of the sham operated rats, and not significantly different from that in the untreated hypophysectomised rats.

The subsequent fall in catabolic rate was less pronounced than that shown by the untreated hypophysectomised rats. Because the synthesis rate fell less slowly after growth hormone treatment the intravascular pool would tend to become depleted at a later stage and hence the compensatory reduction in catabolism would also be delayed. The presence of normal catabolic rates for a full seven or eight days after hypophysectomy strongly suggests that pituitary hormones are not essential for the maintenance of normal catabolic rates.

It is difficult to provide an explanation for the fall in albumin concentration in the growth hormone treated rats, since the concentration falls at a time when the synthesis rate is at its highest and rises when the synthesis rate falls. The disparity between the synthesis and catabolic rates cannot account for the reduced concentration. Since the synthesis rates in the growth hormone treated and sham operated rats were no different on day 2 and the latter showed no fall in concentration, it would seem that the decreased concentration is probably the result of some temporary disturbance of albumin distribution, though the exact nature of this is difficult to understand. Direct measurements of the plasma volume have excluded the possibility of plasma volume expansion causing the hypoalbuminaemia. Arising out of this observation is the possibility that the decreased albumin concentration after hypophysectomy (which was of the same magnitude as the hypophysectomised rats receiving growth hormone) may also be due in part to altered albumin distribution.

MODE OF / .....

MODE OF ACTION OF GROWTH HORMONE

(a) Albumin anabolic or anti-catabolic?

Hypophysectomy results in an immediate cessation of growth and treatment with suitable preparations of growth hormone causes growth to be resumed. However one chooses to define growth it is clear that protein synthesis is intimately concerned in the process. In Chapter 1 earlier evidence was cited in which growth hormone treatment was found to lower plasma amino acid levels, to diminish urinary nitrogen excretion and increase the proportion of protein in the tissues. In view of the dynamic nature of the processes involved in protein metabolism, the increase in protein content of the tissues could have arisen from an increase in protein synthesis or a reduction in protein catabolism. A consideration of the evidence relating to the effect of growth hormone on total body protein turnover falls outside the scope of this thesis suffice it to say that much of the evidence (see references 144, and 214 for review) is as conflicting as that relating to albumin metabolism in which anabolic and anti-catabolic actions have been proposed.

In the present study treatment with growth hormone was shown to result in higher rates of both albumin synthesis and catabolism when compared with untreated hypophysectomised rats, and this latter finding contrasts with the results of those workers who found that growth hormone lowered albumin catabolism. On grounds of commonsense alone it seems highly unlikely that growth hormone could regulate albumin levels effectively if under its influence both synthesis and catabolism were raised simultaneously. It is more reasonable to propose that the action of growth hormone is to stimulate albumin synthesis. This will result in more albumin entering the intravascular pool, the size of which in turn will dictate the rate at which catabolism is to proceed. In this way growth hormone would effect albumin synthesis primarily, and its effect on catabolism would be secondary by virtue of its ability to influence the size of the intravascular pool.

It seems reasonable to extend this proposal to include all the other hormones which stimulate albumin synthesis. Were this the case then albumin synthesis alone

would be controlled / .....

would be controlled on a hormonal basis and catabolism would respond appropriately to any change in the intravascular pool size that had been brought about by changes in the synthetic process. In an attempt to demonstrate the existence of such a homeostatic mechanism a number of hypophysectomised rats were infused with albumin and the catabolic rate measured. It was anticipated that synthesis rates would be low because of the combined effects of the absence of pituitary hormones and inadequate diet, but catabolism would proceed at normal rates because the intravascular pool was being maintained artifically by the albumin infusions. The results of these experiments were disappointing to say the least. The hypophysectomised rat is clearly unable to withstand the repeated stresses of daily albumin infusions, and many rats succumbed to minor allergic reactions. Hypervolaemia may also have contributed to the alarming mortality rate since the rats were infused daily with large doses of albumin (150 - 200 mg), and in the absence of corticosterone, free water clearance is reduced and the ability to deal with a fluid load is impaired. In the few rats that did survive, daily albumin infusions given in a dose sufficient to maintain the intravascular pool within normal limits, and in the absence of proteinuria, maintained the catabolic rate at the upper limit of normal for five or six days after it would usually have been significantly reduced after hypophysectomy. Unfortunately for technical reasons no synthesis rate values were obtained. This experimental approach offers hope of further clarifying the role of the anterior pituitary hormones in albumin metabolism, but is beset with formidable obstacles.

If then it is possible for albumin catabolism to proceed at normal rates without the direct action of the pituitary gland, reconciliation with the known albumin catabolic effects of adrenocortical and thyroid hormones is necessary. The issue is largely speculative but it seems reasonable to suggest that under normal circumstances the size of the intravascular albumin mass controls the catabolic rate as a specific control mechanism, whilst the ability of glucocorticoids and thyroid hormones to stimulate albumin catabolism is merely one of their numerous and widespread actions, which under conditions of hypersecretion may result in excessive albumin catabolism. The central role of the intravascular pool in controlling catabolism is conceptually satisfying. That catabolism proceeds as a first order process geared to the size of the intravascular albumin pool is generally held to be true, although under certain patho-

logical conditions / .....

logical conditions (gastrointestinal, [233] renal [234 - 236] and endocrine [190]) this first order relationship may not hold since high rates of catabolism may exist in the presence of a reduced intravascular pool.

(b) Can growth hormone exert its protein anabolic effect on liver directly?

Young has long held the view that growth hormone achieves its anabolic (nitrogen retaining) properties by stimulating the secretion of insulin from the pancreas, which is held by him to be the protein anabolic hormone, and detailed reviews of the relevant literature have been presented [33, 215, 217]. Later after it was shown that growth hormone did not stimulate the release of insulin from rabbit pancreas [218], it was suggested that growth hormone stimulated the release of insulin bound in some inactive form to muscle. [233] The administration of growth hormone evokes a rise in plasma non esterified fatty acids [219, 220] and with the observation that this rise in fatty acids inhibits the peripheral utilisation of glucose [221] in muscle it remains possible to retain the suggestion that growth hormone acts by stimulating insulin secretion. Thus it has been proposed [222] that growth hormone stimulates lipolysis, the resultant increase in plasma free fatty acids inhibits the peripheral utilisation of glucose thereby causing a rise in blood glucose levels which in turn triggers the secretion of insulin. Insulin has undoubted anabolic properties (see ref 224 for review) and insulin treatment increases [224] and alloxan diabetes reduces [225] the microsomal incorporation of amino acids into protein in the liver cell free system. Yet Jefferson and Korner [226] using a perfused liver system have produced evidence that growth hormone can stimulate protein anabolism in the liver directly and without the mediation of insulin. However 'in vivo' the possibility cannot be excluded that part of the effects of growth hormone treatment are mediated by insulin.

(c) Intracellular action of growth hormone.

The exact mechanism (mechanisms) by means of which growth hormone achieves its undoubted protein anabolic properties to a large extent remains unsolved. Growth hormone increases amino acid uptake into cells [228] and stimulates the ability of microsomes to incorporate amino acids into protein [174, 227] but there is evidence to both support and refute the suggestion that its action is at a genetic level. A consideration of this field of investigation falls outside the scope of this thesis (see refs. 229 - 232 for extensive reviews).

The results of / .....

The results of the experiments conducted in this thesis have shown that hypophysectomy reduces and growth hormone partially restores, both albumin synthesis and catabolic rates, and further that these changes cannot be explained on the basis of a reduction in food intake alone. The growth hormone data presented has been explained by proposing that the action of this hormone is primarily to stimulate albumin synthesis and that catabolism is affected through secondary alterations in the intravascular albumin mass. This proposal though in itself reasonable and indeed conceptually satisfying, provides neither an immediate nor a complete answer as to how albumin homeostasis is achieved on a day to day basis. It is clear from the large volume of experimental evidence that has accrued over the past fifteen or twenty years, that the mechanisms by means of which albumin metabolism is controlled cannot be explained simply. Normal albumin synthesis and catabolism is contingent upon the presence of an adequate supply of amino acids and upon normal gastrointestinal and hepatic function. Also these processes are influenced by the distribution of albumin between the intra and extravascular spaces and by the actions of a number of hormones.

The results of the present experiments led to the suggestion that, in health at least, albumin synthesis might be controlled by hormones alone, whilst catabolism would be effected by a mechanism dependent on the intravascular albumin mass. Clearly this explanation is too simple. Each of the hormones proven or presumed to be capable of stimulating albumin synthesis has numerous other actions. One needs to contemplate the consequences on albumin synthesis if there is a call to hypersecretion of a hormone in order to effect some other metabolic adjustment. For instance, would the rise in growth hormone in response to hypoglycaemia result in a parallel stimulation of albumin synthesis? It seems likely that a more specific system exists in order to prevent unnecessary albumin synthesis. The colloid osmotic pressure in the hepatic interstitium may be such a mechanism. Although Tavill et al [120] and Hoffenberg [142] were unable to depress albumin synthesis after albumin infusions, Rothschild showed that albumin synthesis was decreased after infusions of both albumin [237] and dextran. [137] Growth hormone or ACTH secreted in order to perform some other metabolic function might in some way be 'blocked' in the liver and prevented from stimulating albumin synthesis by the presence of a normal hepatic

colloid osmotic / .....

colloid osmotic pressure when the body albumin mass was normal. One may speculate that a fall in colloid osmotic pressure when the albumin mass was being compromised for one or other reason, might serve as a stimulus to the secretion of the albumin anabolic hormones.

In disease states the picture becomes perplexing. Synthesis and catabolism will of course be affected if severe enough by disease of any of the viscera responsible for these processes, but in addition disease of the endocrine glands, as well as of those viscera responsible for the degradation and elimination of their hormones, may be of consequence for albumin metabolism. The nature of the changes in synthesis and catabolism will of course depend on the particular patholgy, as will the nature of the adaptive changes in albumin metabolism. Clearly a number of different responses are possible according to the disease, and the plethora of events may be impossible to unravel.

Discussion has been largely restricted to a consideration of the hormonal control of albumin metabolism. The interactions of the endocrine system and the widespread and multiple effects on intermediary metabolism, which in turn may give to secondary responses, prevent the formulation of any rigid unitary hypothesis. It is of course tempting to explain the action of hormones in simple terms, and indeed Korner [222] has wryly commented on the psychological need to do so. The available evidence does not justify the occupation of this rather more comfortable position.

CHAPTER 6

SUMMARY

This thesis investigates the role of the anterior pituitary gland, and of growth hormone in particular, in regulating albumin metabolism.

In order to gain historical perspective, Chapter 1 was devoted to a brief review of earlier literature. The first half of the chapter was concerned with a review of the work which first suggested and later confirmed the integral role of the pituitary gland in the regulation of body growth. The potent growth promoting properties of the pituitary gland came to be identified with a factor named simply pituitary growth hormone, and further experimentation clearly implicated this hormone in the regulation of protein metabolism. Plasma albumin was the particular body protein chosen for study in this thesis, and the second half of the chapter was devoted to a review of the effects that hypophysectomy and hypophysectomy and growth hormone replacement therapy have been shown to have on albumin metabolism. Earlier workers demonstrated clearly that hypoalbuminaemia was a regular consequence of hypophysectomy, but studies designed to define the underlying changes in the synthesis and catabolism of this protein were few and contradictory. The role of growth hormone in albumin metabolism had been investigated in both hypophysectomised and intact subjects and these studies were wholly inconclusive. Growth hormone was shown to have little or no effect on plasma albumin levels, and when kinetic studies were undertaken some investigators claimed that the action of this hormone was to stimulate anabolism whilst others believed it to reduce catabolism.

Chapter 2 presented a general account of the principal methods employed in this thesis viz. hypophysectomy, tracer labelling of plasma albumin and the measurement of albumin catabolism and synthesis. Less commonly used methods of hypophysectomy were briefly described but details of the parapharyngeal method, that used in the present study, was deferred and is presented in the Appendix. Tracer labelling of plasma albumin was achieved by using ^{125}Iodine or ^{131}Iodine. Particular emphasis was placed on describing the conditions that have to be met for reliable labelling of plasma albumin, and tests of the satisfactory biological behaviour of the tracer "in

vivo"/.....

vivo" were described. Details of the actual technique of labelling are presented in the Appendix.

The various methods of measuring albumin catabolism were next described, together with a brief account of the compartmental systems through which albumin is believed by various workers to be distributed in the body. The difficulties of certain forms of measurement of catabolism were alluded to, but it was pointed out that catabolism may be reliably measured under steady and non steady metabolic conditions by using urinary radioactivity and plasma specific activity data.

The problem of measuring albumin synthesis under conditions of metabolic change has been overcome by the use of the ^{14}C carbonate method. Whereas only indirect information about synthesis could be gained from the use of iodine labelled tracers, the ^{14}C carbonate method measures synthesis directly and independently of any catabolic measurements performed with iodinated preparations. The historical background and evolution, rationale, and assumptions of the method were described in detail. Recently certain modifications to the method have been suggested in order to achieve greater accuracy of measurement. Practical considerations precluded the implementation of some of these refinements, and the error associated with their deletion was assessed. The laboratory technique is described in outline in Chapter 3, whilst full details are provided in the Appendix. The chapter was concluded with a brief account of the various techniques of measuring albumin synthesis indirectly with iodinated albumin preparations.

The first part of Chapter 3 contained a description of the materials and methods employed in the thesis. For those methods fully described in the Appendix, only a general description was provided in this chapter, but full details were presented of all remaining methods. The second part of this chapter explained the experimental protocol and also discussed the factors which influenced the choice of this particular experimental design. Albumin metabolism was studied in three groups of rats. Changes in albumin synthesis and catabolism were measured in a group of hypophysectomised rats, in a group of hypophysectomised rats receiving daily injections of growth hormone, and in a control group pair fed to the hypophy-

sectomised rats / .....

sectomised rats and subjected to a sham operation. Measurements were carried out for nine days after hypophysectomy or sham operation.

Chapter 4 set out the results of these experiments. The amount of food consumed by the hypophysectomised rats was measured and confirmed the well-known fact that this animals food intake is markedly reduced. Growth hormone administration had no significant effect on this anorexia. Values of albumin synthesis and catabolism in the normal control rats were then presented and compared with values obtained by other workers. In general, satisfactory agreement was demonstrated.

In the hypophysectomised rats eating ad libitum synthesis rates were significantly reduced forty-eight hours after the operation, and were further reduced when measured seven days later. Catabolism however showed a rise above normal levels forty-eight hours after the operation but then fell to abnormally low values. This fall occurred some three or four days later than the fall in synthesis rate.

The sham operated rats which were pair fed to the hypophysectomised rats also showed a marked reduction in synthesis rates forty-eight hours post-operatively, and the values obtained in this group were not significantly different from the values observed in the hypophysectomised rats. However, when provided with more food at the end of the study period, the sham operated rats began to synthesise albumin at normal rates whereas synthesis in the hypophysectomised rats became further reduced. The post-operative rise in catabolism was much greater in the sham operated rats than in the hypophysectomised rats, and unlike the latter did not fall to subnormal levels.

The synthesis rate when growth hormone was administered to hypophysectomised rats was at all times significantly greater than the synthesis rate in the untreated rats. It was observed that the synthesis rate fell progressively during the nine day study period so that whereas half the fractional values fell within the normal range two days after hypophysectomy, all values were below normal at the end of the study period. These rats behaved similarly to the untreated hypophysectomised rats insofar as the synthesis rates fell progressively despite the increase in food consumption. The post-operative increase in catabolism was significantly less than that of the sham operated

rats, / .....

rats, being of the same magnitude as the untreated hypophysectomised rats. Thereafter catabolism was observed to fall to subnormal levels seven or eight days after hypophysectomy, some two to three days later than the time when this fall had already occurred in the untreated rats. Growth hormone was thus observed to increase both synthesis and catabolic rates.

In Chapter 5, the results of the experiments were discussed and explained. The pair fed data was dealt with first and was compared with data obtained by other workers. It was confirmed that the regulation of albumin synthesis was intimately related to the availability of substrate amino acids, whereas catabolism appeared to proceed as a first order process based on the size of the intravascular albumin pool. The basis of the hypercatabolic post-operative response was discussed and it was concluded that at least part of this response was mediated by the pituitary gland, and was most likely to be due to excess A.C.T.H. secretion.

From the data obtained, it was concluded that the early reduction in synthesis rate after hypophysectomy was no greater than could be accounted for by a reduction in food intake. Later measurements however clearly indicated that hypopituitarism was contributing to the reduction in synthesis rate. The fall in catabolism was ascribed to a reduction in the intravascular albumin mass, although it could not be stated with certainty that falling hormonal levels were not contributary. From a consideration of the sham operation and hypophysectomy data together, it was concluded that part of the adaptations that occur in albumin metabolism during changes in dietary protein intake were mediated by the pituitary gland.

Growth hormone was shown to increase the rate of albumin synthesis. Rates of synthesis however fell below the normal range and it was concluded that the poor food intake was one limiting factor. It appeared likely that glucocorticoid and thyroid hormones are necessary for normal albumin synthesis and that their absence also prevented the restoration of normal synthesis rate levels. The fall in catabolism was more confidently ascribed to a shrinking intravascular albumin mass than was possible for the untreated hypophysectomised rats.

The mode of / .....

The mode of action of growth hormone was then discussed and it was proproposed that the action of this hormone is to stimulate albumin synthesis, while any effects on catabolism are indirectly achieved through alterations in the intravascular albumin mass. The concept was extended to include all albumin anabolic hormones and it was speculated that in health at least, albumin synthesis was hormonally controlled whereas catabolism was controlled by the intravascular pool size. Experiments undertaken to prove this were however unsuccessful.

APPENDIX

I. STATISTICAL METHODS

(1) Mean. $\bar{x} = \frac{\sum x}{n}$

Standard deviation of the mean. $S = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$

Standard error of the mean. $e = \frac{S}{\sqrt{n}}$

(2) Linear regression analysis (Method of Least Mean Squares)

The least square line approximating the set of points (x_1, y_1) (x_2, y_2) ..... (x_n, y_n), is given by the equation $y = mx + c$ where

$$m = \frac{N\sum xy - (\sum x)(\sum y)}{N\sum x^2 - (\sum x)^2} \qquad \text{and } c = \frac{(\sum y)(\sum x^2) - (\sum x)(\sum xy)}{N\sum x^2 - (\sum x)^2}$$

(3) Testing whether two independent groups have been drawn from the same population.

For the numerous comparisons undertaken between the various sets of experimental data the "significance of the difference" was tested by means of the Mann-Whitney 'U' test. This non-parametric test is an excellent alternative to the parametric Students' 't' test, since no assumptions are made about either the distribution or the variances of the populations from which the samples are drawn.

$$U_{(obs.)} = n_1 n_2 + \frac{n_1 (n_1 + 1)}{2} - R_1 \text{ ---------- (1)}$$

or equivalently, $U_{(obs.)} = n_1 n_2 + \frac{n_2 (n_2 + 1)}{2} - R_2$ ---------- (2)

where R_1 = sum of the ranks assigned to the group whose size is n_1

R_2 = sum of the ranks assigned to the group whose size is n_2

and where $n_2 \geq n_1$

Where formulae (1) and (2) yield different values for 'U' select the smaller of the two.

When both /

When both n_1 and n_2 lie between 3 and 8, the exact probability (p) associated with the occurrence under Ho of any U as extreme as an observed U(U obs) may be determined. When n_2 lies between 9 and 20 exact probabilities cannot be determined. In this instance critical values of U are provided from tables at different significance levels. Where U (obs) is less than or equal to the critical value of U, Ho may be rejected at the level of significance chosen i.e. the difference between the two groups is significant at that level.

Wherever possible in the presentation of the data, exact probabilities have been provided. Where sample sizes precluded this the observed value for U is presented together with the critical value of U at the .001 and .01 significance levels.

For example: U(obs) = 8. From tables: Critical value of U at the .001 level = 4
Critical value of U at the .01 level = 12

Then the two groups that were compared are significantly different at the .01 level, but not at the .001 level since 8 is less than 12 but greater than 4. In presenting values of U the following convention (as illustrated by the figures in the above example) was employed: $(8\ {}^{4}_{12})$

Significance was assessed at the following levels:-

p	$\leq$ .05	-	Probably significant
p	$\leq$ .01	-	Significant
p	$\leq$ .001	-	Highly significant

II SPECIFICATIONS FOR GROWTH HORMONE, BOVINE, NIH-GH-B14

(as provided by the National Institutes of Health)

GROWTH HORMONE ACTIVITY:

Growth hormone activity was determined by the ten day body weight gain test in 100 gm. hypophysectomised female rats (1), using the subcutaneous injection

modification (2) / .....

modification (2). USP Growth hormone was used as the reference preparation. The unweighted geometric mean potency of NIH-GH-B14, obtained from five replicate assays, was 1.04 USP units per mg.

CONTAMINATION WITH OTHER PITUITARY HORMONES:

THYROID STIMULATING HORMONE (TSH) ACTIVITY: TSH Activity was determined by the P-32 uptake method using baby chicks (3). USP TSH was used as the reference preparation. The unweighted geometric mean potency obtained from two assays was 0.0021 USP units/mg.

LUTEINISING HORMONE (LH) ACTIVITY: LH Activity was determined by the ovarian ascorbic acid depletion assay (4) (7) NIH-LH-S1 was used as the reference preparation. The unweighted geometric mean potency obtained from two assays was 0.0017 NIH-LH-S1 units/mg. (One unit = activity in one mg. of NIH-LH-S1.)

FOLLICLE STIMULATING (FSH) HORMONE ACTIVITY: FSH Activity was determined by the HCG-augmentation method (5). NIH-FSH-S1 was used as the reference preparation. A total dose of 7200 micrograms of NIH-GH-B14 failed to elicit a significant response. Therefore, this preparation is judged to contain a maximum of 0.0097 NIH-FSH-S1 units/mg. (One unit = activity in one mg. of NIH-FSH-S1.)

PROLACTIN ACTIVITY: Prolactin activity was determined by the pigeon crop-sac weight method (6). The results are expressed in terms of the 2nd International Standard for Prolactin. A total dose of 12 mg. failed to elicit a significant response in this assay. Therefore, NIH-GH-B14 is judged to contain a maximum of 0.42 I.U/mg. prolactin activity.

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Parlow / .....

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III METHODS

A Hypophysectomy:

Provided the colony from which the rats were drawn had received the customary attention to feeding and hygiene, no special pre-operative measures were found to be necessary. On the morning of the operation food and water was withheld in order to prevent regurgitation under anaesthesia. The rat was placed in a bell jar and anaesthesia induced with Halothane at a concentration of 3.5%, oxygen being provided at a flow rate of four litres per minute. Induction was rapid and uniformly satisfactory.

Once anaesthesia had been induced the area for incision was shaved and the rat returned to the bell jar. At this stage the Halothane was reduced to a concentration of 1% and gauze swabs moistened with ether were introduced into the bell jar in order to deepen the level of anaesthesia. Using the pattern of respiration as a guide to the level of anaesthesia, when the plane of surgical anaesthesia was reached, the rat was removed from the bell jar and placed on an operating board on its back with its head towards the operator, and immobilised in this position by means of rubber bands looped about the legs. The head and neck were extended by a firm rubber band passed over the upper incisors. Anaesthesia was maintained by ether swabs placed near the mouth and nostrils of the rat.

A skin incision was placed in the midline of the neck extending for two to three centimetres caudad to the mandibular papilla and the underlying salivary glands and lymph nodes separated in the midline by blunt dissection. The sternohyoid muscles were then split in the midline to expose the trachea in preparation for the insertion of a tracheostomy, (photograph 1) for in gaining adequate exposure of the

site for drilling / .....

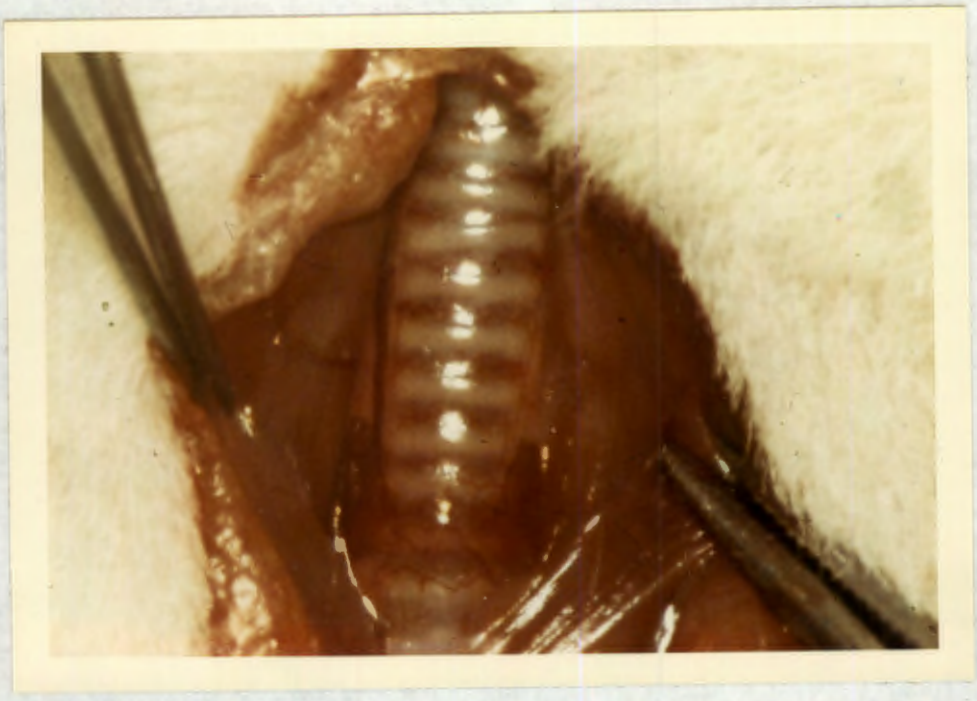
site for drilling, the respiratory passages are completely blocked and respiration is impossible unless a tracheostomy is provided. The closed tip of a fine pair of forceps was passed through the right omohyoid muscle near its posterior border, and then opened in order to split the muscle longitudinally for one and a half to two centimetres. The forceps was then passed behind the oesophagus and was pushed through the left omohyoid muscle. The surgical plane for the placing of a staying suture (see following paragraph) was conveniently defined by allowing the forceps to remain in this position while the tracheostomy tube was inserted.

A sharp pointed scalpel blade was used to incise the trachea, (photograph 2). In order to prevent bleeding the space between the two cartilagenous rings with the least number of visible capillaries was chosen for incision. A small length of soft plastic tubing (infant nasal feeding tube, size 5 French was found to be suitable) was inserted (photograph 3) and secured in place by a staying suture passed not only around the trachea, but also encompassing the strap muscles, and the oesophagus. Once the staying suture was tied anteriorly the forceps which had assisted in its positioning was removed. Since the oesophagus, trachea and strap muscles were contained within a single suture, they were conveniently retracted laterally to the left by means of a single retractor, thereby exposing the prevertebral muscles (longus colli) and the base of the skull. (photograph 4). More favourable exposure was obtained by the use of additional retractors to draw the sternomastoid muscles of each side laterally.

Photograph 1 / .....

Photograph 1

The sternohyoid muscles have been split in the midline and retracted laterally to expose the trachea. The thyroid gland is clearly visible at the proximal end of the trachea.

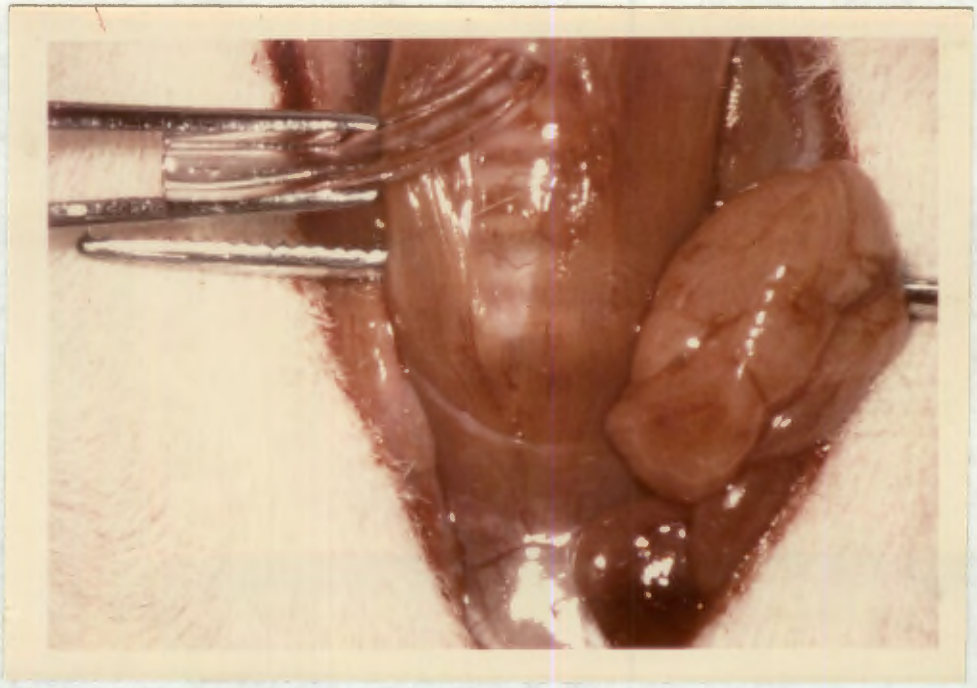


Photograph 2

Tracheotomy is performed by means of a sharp pointed scalpel blade. The trachea overlies and partially obscures the pair of forceps used to define the surgical plane of a staying suture.

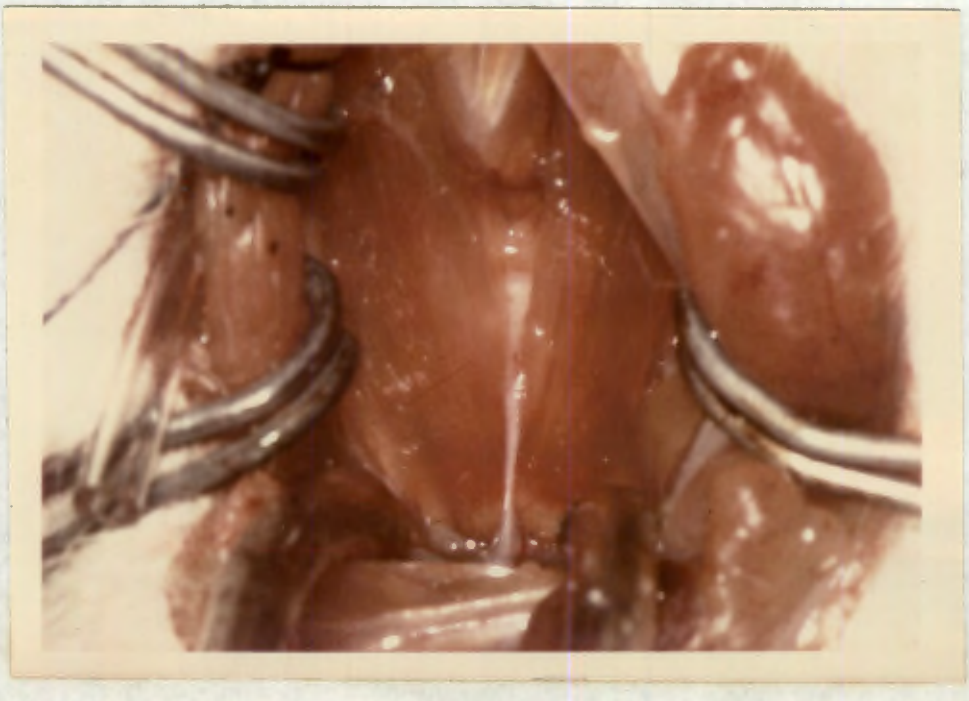
Photograph 3

The tracheostomy tube is shown in position. The submandibular salivary gland and a lymph node are visible to the right of the trachea.



Photograph 4

The trachea, oesophagus and strap muscles have been retracted laterally to the left. Additional lateral retractors have drawn the sternomastoid muscles aside, and a curved forceps has been used to retract the free border of the anterior digastric muscles in a cephalad direction. The muscles visible in the centre of the operating field are the longus colli which overlie the basi-occiput. The two muscles can be seen to be separated by a fibrous membrane which has attachments to the basi-occiput, and to the posterior pharyngeal wall (the latter attachment not visible in this photograph). The spheno-occipital synchondrosis can be seen as a faint line deep to longus colli muscles near their apex (between the blades of the curved forceps in this photograph).



From this point on, the operation was carried out with the help of an operating microscope, and a good source of illumination. A magnification factor of six was found to be most suitable. This author was fortunate enough to have the use of an instrument which projected the source of light through the magnifying lens. Presumably equally satisfactory results would be obtained with less sophisticated apparatus.

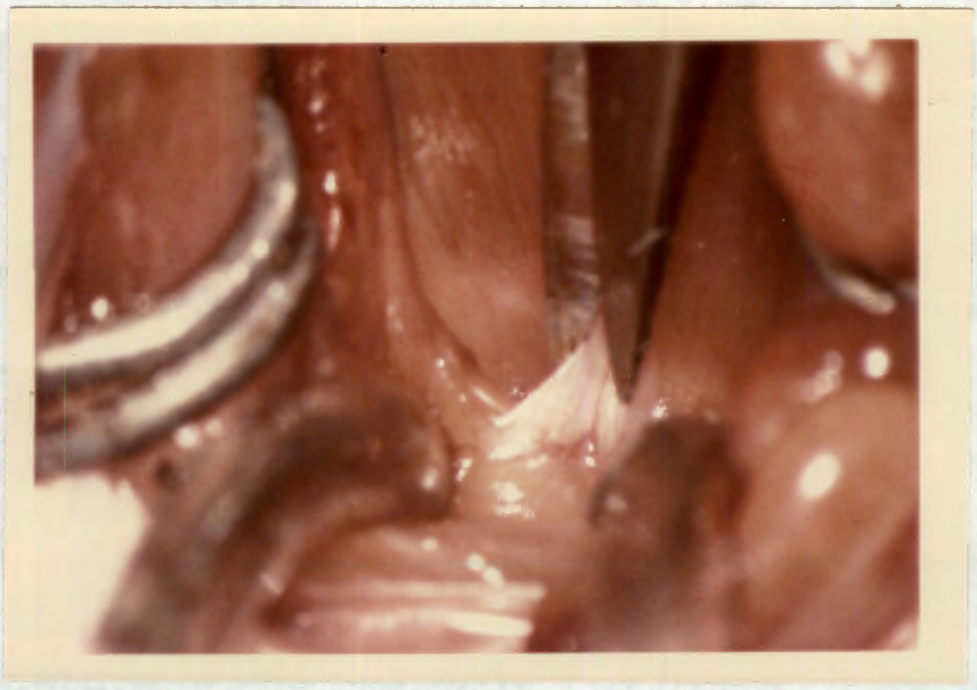
A small curved forceps was introduced against the free border of the anterior belly of the digastric muscles, and moderate traction cephaled exposed the area to be drilled (photograph 4). The posterior wall of the pharynx, lying just deep to the digastric muscles, became visible along its entire attachment to the base of the skull, and the firm fibrous band running from it to the prevertebral muscles was cut (photograph 5). The fact that the pharyngeal insertion is linear has considerable bearing on the subsequent steps to be taken in the operation, as will be outlined below. The longus colli muscles gain a flat insertion on the basi-occiput and terminate in juxaposition to the pharynx at the spheno-occipital synchondrosis. This synchondrosis provides the landmark for placing the drill bit.

The linear attachment of the pharynx lies directly over the intracranial site of the pituitary gland and access may therefore be gained by drilling on either side of the pharyngeal wall, i.e. from either within the nasal cavity, or from without, viz. from the neck. The main departure here from the method used by Smith, [12] is in respect of the approach to the pituitary gland. That author incised the pharyngeal wall and gained access to the gland through the roof of the nasal cavity. The method used here leaves the pharynx intact by approaching the gland from the neck. Experience has shown that if the linear attachment of the pharynx is disturbed, and artificial opening is created between the nasopharynx and the neck muscles through which the animal breathes once the tracheotomy is closed. This leads to intense respiratory difficulties, and a fatal outcome within a few hours is invariable. Therefore when scraping away the longus colli muscle from its attachment to the occiput, a small fringe of this muscle was left intact to act as a reinforcement to the pharyngeal insertion (photograph 6 and 7).

Photograph 5 / .....

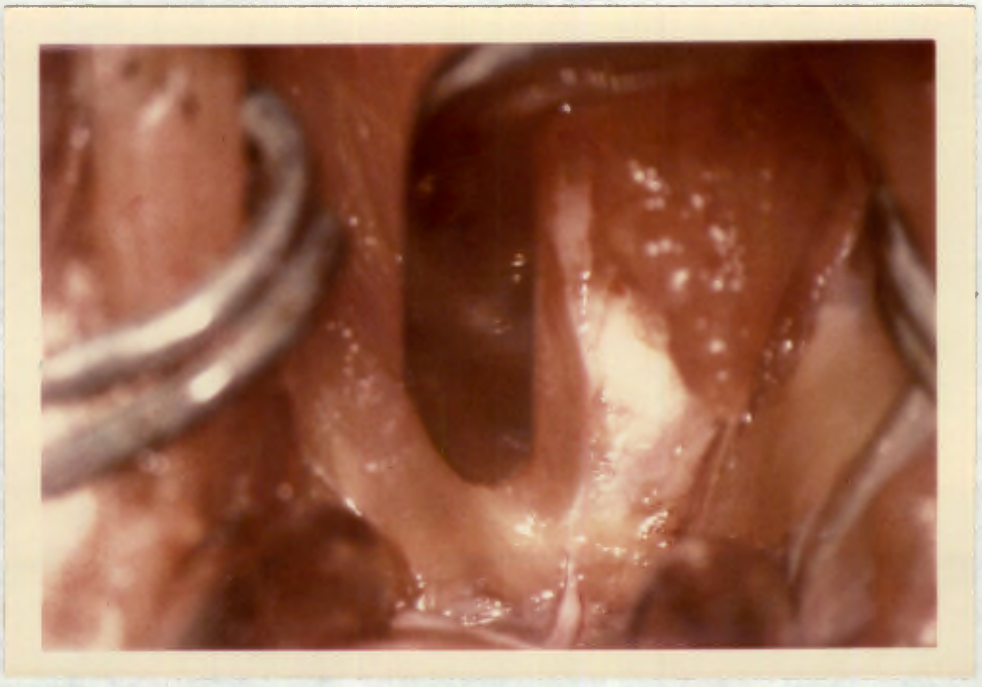
Photograph 5

The fibrous band running between the posterior pharyngeal wall and the base of the skull is cut.



Photograph 6

The longus colli muscles are scraped off the base of the skull, with a dental periosti um elevator. The right longus colli muscle has been stripped of it's attachment, and the left longus colli is in the process of being stripped.



Once the muscle overlying the synchondrosis had been scraped free, an electric dental drill operated by a foot pedal was used to drill the base of the skull (photograph 7). An angled handpiece and a No. 6 round headed ash bur was found to be most suitable for the size of the rat used in this work. The drill was placed vertically over the synchondrosis, and a vertical direction of drilling maintained throughout the thickness of the skull base. Since the synchondrosis is not itself vertical but slopes somewhat, maintaining a vertical path in drilling will ensure that the drill bit comes to be completely cephalad to the synchondrosis. The complete thickness of the skull may be drilled, or the innermost table of bone picked clear with a dissecting needle. If the former method is chosen, care must be taken to determine if both lobes of the gland have not been pushed to one side of the hole by the rotating action of the drill bit. Failure to do so may result in incomplete aspiration of the gland.

A finely drawn out glass pipette was inserted into the hole and the gland aspirated by means of a mechanical aspirator (photographs 8, 9 and 10). The three lobes of the gland were always identified inside the glass pipette. By adopting this procedure, a complete hypophysectomy was assured since experience had shown that the lobes never fragment.

Debris was cleared away and the retractors removed. The tracheostomy tube was removed and any mucous and blood that had accumulated in the trachea was gently aspirated. The tracheotomy was closed with a single silk suture (5'0' on an atraumatic round bodied needle). Skin closure was obtained with three or four small surgical clips or silk sutures.

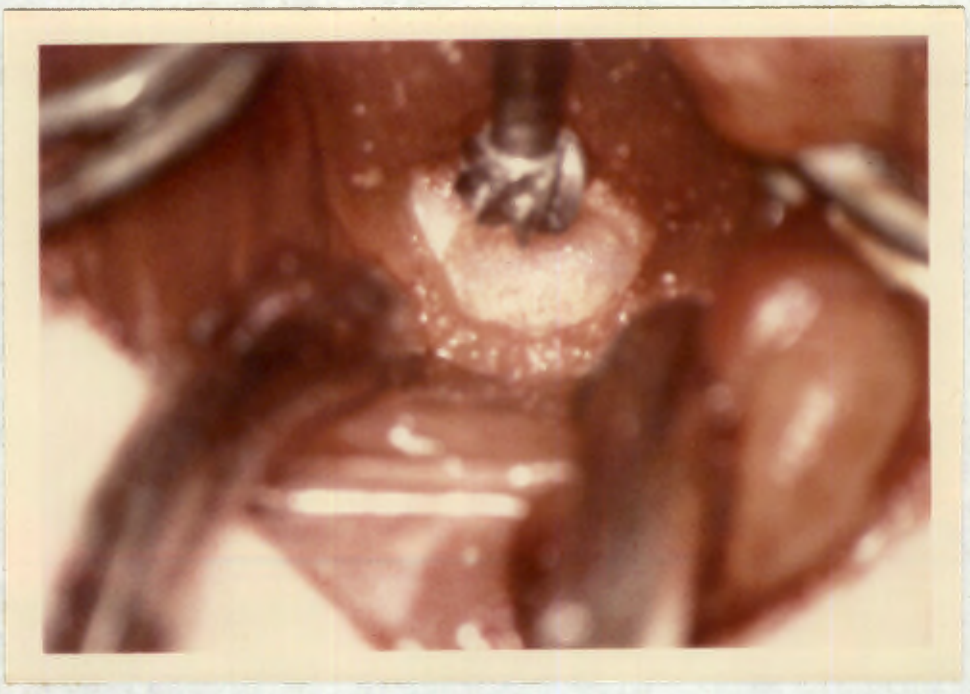
Recovery from the anaesthetic was usually complete within ten to fifteen minutes after completion of the operation. The cages were kept dry by frequent changes of newspaper bedding and drinking water was generously provided.

In the rat the absence of any firm attachment of the gland to its sheath, and the presence of a strong diaphragma sellae, prevent any damage to the hypothalamus when the gland is aspirated. The rat exhibits only a temporary diuresis for one to two days since the pituitary stalk becomes revascularised and neurohypophyseal function returns. Once experience had been gained, the operation not lasting longer than ten minutes, a survival rate of approximately 60% was achieved.

Photograph 7/ .....

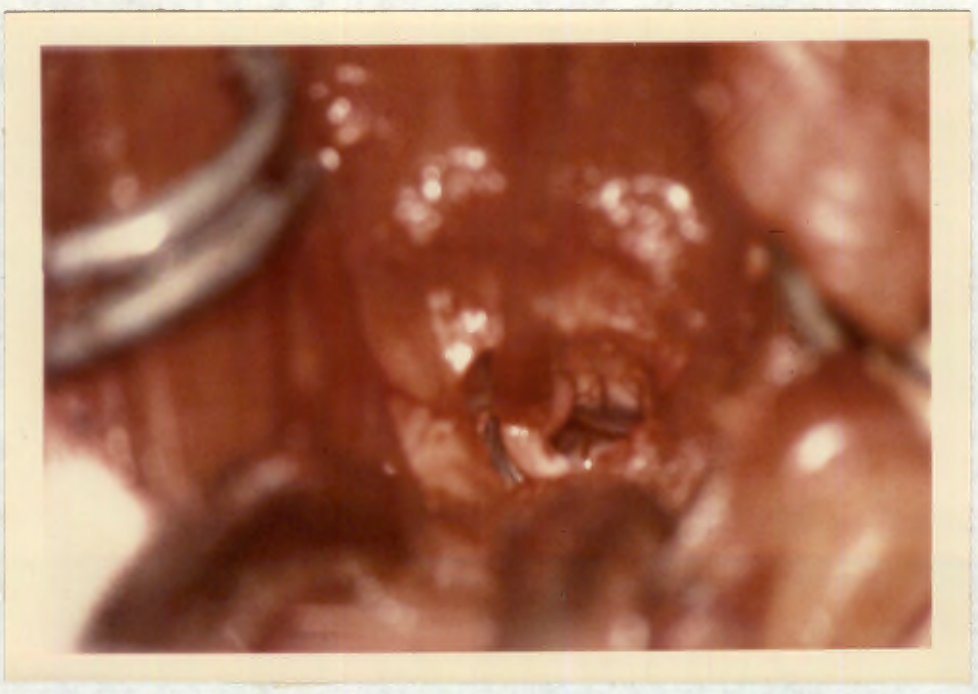
Photograph 7

The base of the skull is drilled at the position of the spheno-occipital synchondrosis which is seen as a faint line running across the drill hole. The cushioning rim of longus colli muscle in juxtaposition to the pharyngeal wall is clearly seen.

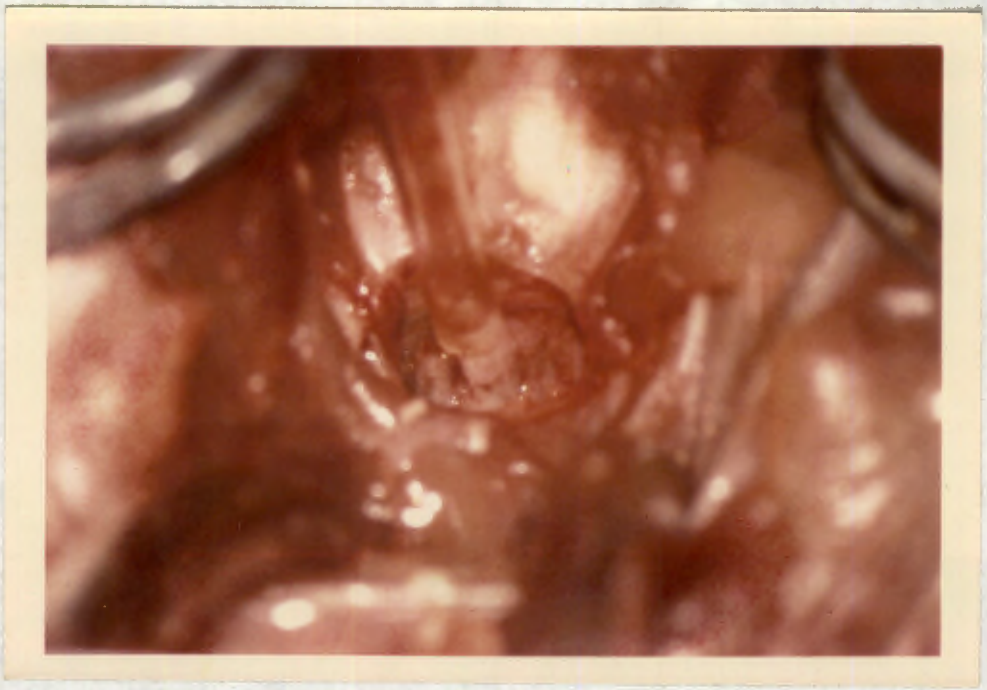


Photographs 8 - 10

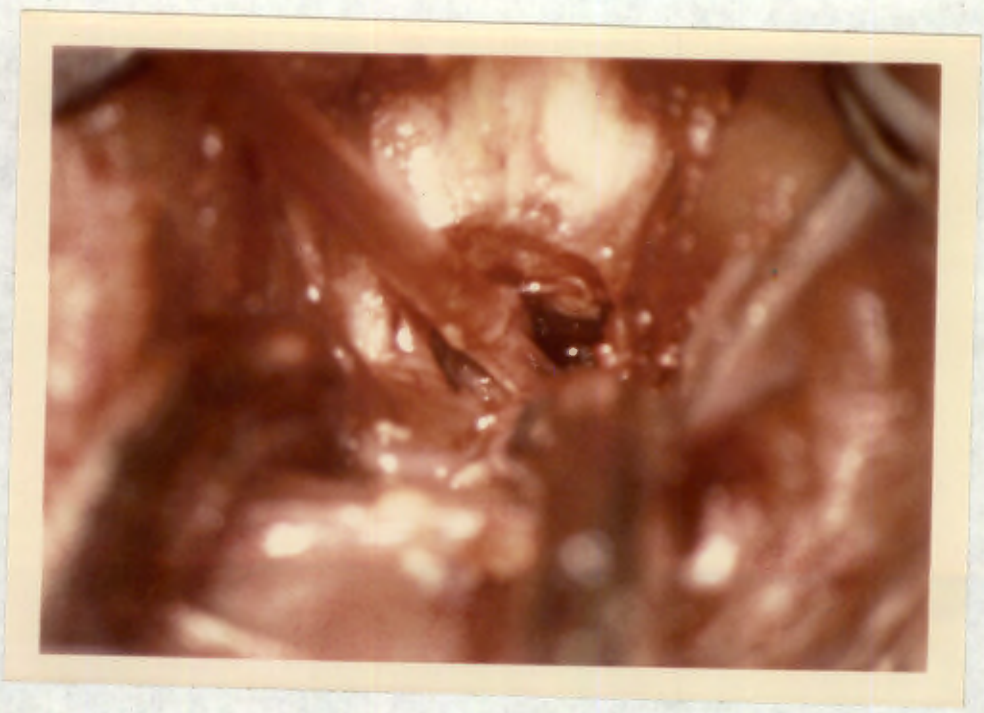
Aspiration of the pituitary gland into the glass pipette.



Photograph 9

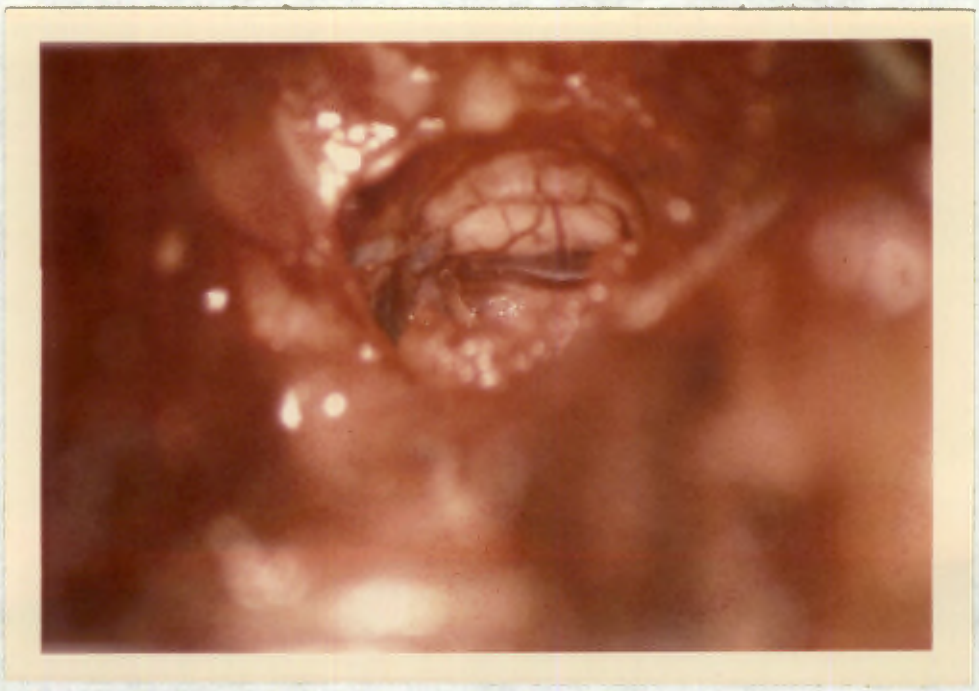


Photograph 10



Photograph 11

After removal of the pituitary gland the base of the brain and some of the blood vessels comprising the Circle of Willis, are clearly visible.



Photograph 12

A composite view of the operating field. The size of the pituitary gland may be compared with that of the head of a matchstick.

B Preparation of Rat Albumin

(a) To one volume of plasma add two volumes of normal saline.

(b) Add three volumes of ammonium sulphate saturated at 37° dropwise with continuous shaking.

(c) Allow to stand for thirty minutes, and then centrifuge to remove globulin precipitate.

(d) Bring supernatant to pH 4.6 with 10% (v/v) acetic acid.

(e) Allow to stand for ten minutes. Centrifuge.

(f) Dissolve albumin precipitate in distilled water. Bring to pH 7.0 with 0.1N sodium hydroxide.

(g) To remove residual salt, dialyse overnight against distilled water at 4°.

(h) Finally check (i) concentration of albumin by biuret method

(ii) purity of fraction by cellulose acetate electrophoresis.

C Determination of Plasma Albumin Concentration

Note. In this thesis, the original method as described hereunder was scaled down for use on half volumes of plasma.

Reagents

1. 0.9% (w/v) NaCl.

2. HCl - ethanol.

To 600 ml. of "denatured" ethanol add 1.0 ml. concentrated HCl (Denatured ethanol is a mixture of 95% absolute ethanol and 5% methanol). Store at 4°.

3. 0.2M Sodium acetate-ethanol.

Dissolve 2.7218 mg. sodium acetate in 5.0 ml. methanol. Add 95.0 ml. absolute alcohol. Store at 4°.

Note. Sodium acetate is soluble in methanol but not in absolute ethanol. Solution must therefore be achieved in methanol prior to the addition of ethanol.

4. 3% / .....

4. 3% (w/v) Sodium hydroxide.

5. Biuret reagent.

Dissolve (i) 17.3 g. Copper sulphate pentahydrate in 100 ml. hot distilled water.

(ii) 173 g. Sodium citrate and 100 g. anhydrous sodium carbonate in 800 ml. distilled water while heating.

When cool, pour (ii) into (i) with rapid stirring. Dilute to one litre with distilled water.

6. Protein standard.

30 mg/ml. aqueous solution of crystalline bovine albumin.

Method

(a) To 0.2 ml. of plasma add 0.8 ml. of normal saline.

(b) Add 9.0 ml. HCl-ethanol dropwise with continuous shaking.

(c) Incubate for thirty minutes at 37°.

(d) Centrifuge.

(e) Transfer 5.0 ml. of the albumin rich supernatatant fluid to another centrifuge tube.

(f) Add 0.5 ml. 0.2M sodium acetate-ethanol dropwise with continuous shaking.

(g) Stopper and allow to stand at room temperature for ten minutes.

(h) Centrifuge. Tip off supernatant. Invert tubes on filter paper to ensure all supernatant fluid is removed.

(i) Dissolve precipitate in 5.0 ml. 3% sodium hydroxide, add 1.0 ml. biuret reagent, and mix by inversion.

(j) Read intensity of colour produced after thirty minutes at 545 μ.

A reagent blank consists of 5.0 ml. 3% sodium hydroxide plus 1.0 ml. Biuret reagent. The known protein standard is made to consist of 4.9 ml. 3% sodium hydroxide, 0.1 ml. of a 3g.% bovine albumin solution, and 1.0 ml Biuret reagent. Colour production was measured on a Zeis PMQII spectrophotometer.

D Iodination

Reagents

1. Approximately 15 mg. of ammonium sulphate prepared albumin is used for labelling. This should be contained in a volume no greater than 0.5 ml.
2. Iodine monochloride (stock solution).
   (i) Dissolve 150 mg. sodium iodide in 8.0 ml. 6N HCl.
   (ii) Dissolve 108 mg. sodium iodate monohydrate in 2.0 ml. distilled water.
   (iii) Forcibly inject (ii) into (i) to avoid precipitation of iodine.
   (iv) Dilute to 40.0 ml. with distilled water and shake with 5.0 ml. carbon tetrachloride.

The presence of a red discolouration in the organic solvent indicates the presence of free iodide. The carbon tetrachloride is removed with a pipette, and the extraction process is repeated until the carbon tetrachloride remains unchanged in colour.

   (v) Bubble moist air through ICl solution for one hour to remove any residual carbon tetrachloride.
   (vi) Make up to 45.0 ml. with distilled water. This stock ICl solution is stored at $4^{o}C$.
   (vii) Before use, dilute one volume of stock solution with nine volumes of 2M NaCl.

3. Glycine buffer (stock solution).
   (i) Dissolve 7.5 g. glycine in 75.0 ml. distilled water.
   (ii) Add 25.0 ml. 1N NaCl.

Alkaline glycine buffer (pH 9.0 - 9.5) is made up prior to use by the addition of 0.2 ml. 1N (w/v) NaOH to 1.8 ml. of the stock solution.

Method

   (a) Prepare alkaline glycine buffer as above.

(b) Prepare / .....

(b) Prepare 1.0 ml. of a working solution of ICl. (0.1 ml. ICl plus 0.9 ml. 2M NaCl).

(c) Place required amount of radioiodine solution in 0.3 ml. working ICl solution. (Tube A).

(d) Bring pH of albumin solution to 8.5 with alkaline glycine buffer. (Tube B).

(e) Likewise bring pH of Tube A to 8.5.

(f) Rapidly inject contents of tube A into tube B by means of a Pasteur pipette.

Note: Once the pH of Tube A reaches 8.5, side reactions, in which radioiodine participates, are initiated. The radioiodine thus involved becomes unavailable for protein labelling. [72] Therefore to ensure maximum efficiency of iodination, as little delay as possible should occur between steps (e) and (f).

(g) If labelled albumin is to be used for synthesis rate studies, remove free iodide by passage through a five centimetre column of Deacidite FF resin in the chloride form.

(h) If labelled albumin is to be used for catabolic rate studies, add 1.0 ml. of fresh rat plasma and carry out ammonium sulphate precipitation and dialysis against distilled water.

(i) In either case check free radioiodide content on a small aliquot (0.01 ml.) of the final preparation by trichloroacetic acid precipitation.

(j) Subject final preparation to cellulose acetate electrophoresis, and ascertain that the label is confined only to the albumin fraction.

(k) Sterilise by "millipore" filtration prior to use.

E. Measurement of Albumin Synthesis

Reagents:

1. 10% (w/v) and 5% (w/v) trichloroacetic acid.
2. Absolute ethanol.
3. CO_2 free 1N NaOH.

(i) Prepare CO_2 free water by boiling 200 ml. distilled water to half its volume.

(ii) To 10.0 ml / .....

(ii) To 10.0 ml. of 10N NaOH (in which CO_2 is insoluble) add 90 ml. CO_2 free H_2O. Stopper tightly and prepare a fresh solution for each batch of samples processed.

4. 4 M Citric acid.

5. Phosphate buffer (pH 6-7).

Dissolve 41.75 g. sodium pyrophosphate and 5.7 ml. orthophosphoric acid in 500 ml. distilled water.

6. Urease solution.

A 1 mg/ml. solution of urease (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.) in phosphate buffer. Stored at 4° and renewed monthly.

7. 10% (w/v) Sodium tungstate.

8. Tungstic acid.

Make up 10.0 ml. 10% (w/v) sodium tungstate and 3.8 ml. 1N HCl to 100 ml. with distilled water.

9. Citric-tungstic acid solution.

A 1 : 1 mixture of 4M citric acid and tungstic acid.

10. 0.66 N Sulphuric acid.

11. Resins: (Deacidite FF resin is obtained commercially in the chloride form).

A. Hydroxide form.

(i) Wash the chloride form with distilled water until eluate is clear.

(ii) Wash with 2 - 3 litres of 4N NaOH.

(iii) Wash with 2 - 3 litres of 2N NaOH.

(iv) Determine if eluate is free of chloride ions by testing with silver nitrate at acid pH.

(v) If necessary repeat washings with 4N and 2N NaOH until eluate is chloride free.

(vi) Wash with distilled water to pH 7.0.

B. Carbonate Form

(i) Wash the hydroxide form with several litres of 0.5N sodium bicarbonate.

(ii) Wash with distilled water to pH 7.0.

C. Mixed form.

A 1 : 1 mixture of chloride and hydroxide forms.

12. Activated / .....

12. Activated arginase solution.

Prepare manganese/maleic buffer.

(i) Dissolve 8.90 g. sodium maleate in 200 ml. distilled water.

(ii) Bring pH to 9.7 - 9.8 with 1N NaOH.

(iii) Add 11.5 g. Manganese sulphate ($MnSO_4.4H_2O$) and make up to 500 ml. with distilled water.

(iv) Bring to pH 7.0 with 1N HCl.

Arginase solution is made up as a 1 mg/ml. solution of arginase (Nutritional Biochemicals Corporation) in manganese/maleic buffer.

Add required amount of arginase to the appropriate volume of buffer, and incubate for three hours at 37°. The solution is stored at 4° and renewed every two months.

13. Phenylethylamine-methanol.

(i) Redistil commercially obtained phenylethylamine until yellow colour is removed.

(ii) Mix with an equal volume of methanol.

(iii) Store in a dark bottle in a lightproof cupboard.

14. Scintillation mixture.

Dissolve 7.5 g. 2,5-diphenyloxazole (P.P.O.) and 0.75 g. 1,4-bis-(4-methyl-5-phenyloxazole-2-yl) benzene (P.O.P.O.P.) in 2.5 litres anhydrous toluene.

METHOD

A. Preparation of samples for measurement of albumin guanidine carbon S.A.

1. Extraction of albumin from plasma

(i) Pool 0.25 ml. plasma from each of the four rats in the batch being studied. To this add 1.0 ml. of a 100 mg/ml. aqueous solution of crystalline bovine albumin.

(ii) Add 2.0 ml. cold 10% trichloroacetic acid.

(iii) Mix well, centrifuge and discard supernatant.

(iv) Wash / .....

(iv) Wash precipitate with 2.0 ml. cold 5% trichloroacetic acid. Centrifuge and discard supernatant.

(v) Add 6.0 ml. absolute ethanol to precipitate. Mix thoroughly and centrifuge to remove globulin precipitate.

(vi) Dialyse albumin rich supernatant against distilled water overnight at 4^{o}.

(vii) Concentrate sample to approximately 2 ml. Confirm homogenecity of fraction by cellulose acetate electrophoresis. Measure the volume and concentration by Biuret method to give the total amount of albumin present.

II Acid hydrolysis of albumin

(i) Given that 100 ml. of 6N HCl is added to 250 mg. protein, determine amount of 6N HCl required for sample (I vii).

(ii) Add volumes of water and concentrated HCl to sample (making allowance for the volume of the sample itself) such that the final solution is 6N with respect to HCl.

(iii) Transfer to hydrolysis tubes and hydrolyse for 16 hours at $110-115^{o}$.

(iv) Transfer hydrolysate to round-bottom flask and evaporate to dryness.

(v) Wash with 3 to 5 ml. distilled water and evaporate to dryness.

III Neutralisation of hydrolysate, and incubation with arginase

(i) Dissolve amino acid residue in 2 to 3 ml. distilled water.

(ii) Bring to pH 7.0 by the addition of Deacidite FF resin in the carbonate form.

(iii) Pass through a sintered glass column containing approximately 5 mm. of the carbonate resin as a filter bed.

(iv) Wash flask with about 10 ml. distilled water and add washings to the column.

(v) Collect eluate in round bottom flask.

If less than 100 mg. albumin was hydrolysed:-

(a) Add 2 ml. activated arginase solution.

(b) Bring to pH 9.0 with 1N NaOH.

(c) Incubate / .....

(c) Incubate for 16 hours at 38°.

If more than 100 mg. albumin was hydrolysed:-

(a) Prepare a column (9 by 1 cm.) of mixed chloride and hydroxide resins. Wash with distilled water to pH 7.0.

(b) Concentrate eluate (III v) to approximately 2 ml. and place on column.

(c) Wash column with distilled water, collecting 4 ml. aliquots of the eluate. Collect six such aliquots.

(d) Spot one drop of each aliquot on filter paper and dry in a hot oven ($\pm$ 110°). Spray with ninhydrin and return to oven until dry. The presence of arginine is indicated by the spot turning a purple colour.

(e) Place those aliquots containing arginine into a round bottom flask, add 3 ml. activated arginase solution, adjust to pH 9.0 and incubate as above.

After incubation

(vi) Bring sample to pH 2.0 with 4 M citric acid.

(vii) Evaporate to dryness.

(viii) Dissolve urea solution in 1 ml. distilled water and 0.5 ml. phosphate buffer. Bring to pH 7.0 with CO_2 free 1N NaOH.

Sample is now ready for gas-train, (Figure A:1)

B. Preparation of samples for measurement of urea carbon S.A.

(i) Pool 0.1 ml. blood from each of the four rats in the batch being studied. Add 2.0 ml. of a 1 mg/ml. aqueous solution of urea.

(ii) Precipitate proteins by adding 1.0 ml. 10% sodium tungstate, and 1.0 ml. .66N sulphuric acid. Mix well and centrifuge.

(iii) Place protein free supernatant in round bottom flask and evaporate to dryness.

(iv) Redissolve in 1.0 ml. distilled water and 0.5 ml. phosphate buffer.

(v) Bring to / .....

(v) Bring to pH 7.0 with CO_2 free 1N NaOH.
Sample is now ready for gas-train. (Figure A:1)

C Measurement of $^{14}CO_2$ S.A.

(i) Place sample in centre bulb of reaction vessel. Place 1 ml. urease in one side-arm and 1 ml. citric-tungstic acid solution in the other side-arm.

(ii) Briefly immerse vessel in a solid CO_2-ethanol mixture and evacuate gasses present in solution during gentle thawing. Seal vessel by closing tap.

(iii) Tip in urease from side-arm and allow to incubate for one hour at room temperature.

(iv) At the end of this time tip in the acid mixture and shake gently to release enzymatically produced CO_2.

(v) Place reaction vessel on gas-train and immerse in solid CO_2-ethanol.

(vi) Evacuate gas line.

(vii) Immerse cold finger in liquid nitrogen and allow CO_2 to pass from reaction-vessel to cold finger.

(viii) Evacuate any contaminant gasses present whilst CO_2 sample is trapped under liquid nitrogen in the cold finger.

(ix) Close tap above cold finger. Thaw cold finger in warm water and then immerse in solid CO_2 - ethanol, thus releasing the CO_2 sample but trapping any contaminant gasses.

(x) Fill cuff surrounding manometer arm with liquid nitrogen and allow CO_2 sample to pass from cold finger to manometer arm by opening the intervening taps.

(xi) Raise mercury column to trap CO_2 sample in the manometer arm and thaw by adding warm water to the cuff. The CO_2 sample is thus released into the modified MacLeod chamber.

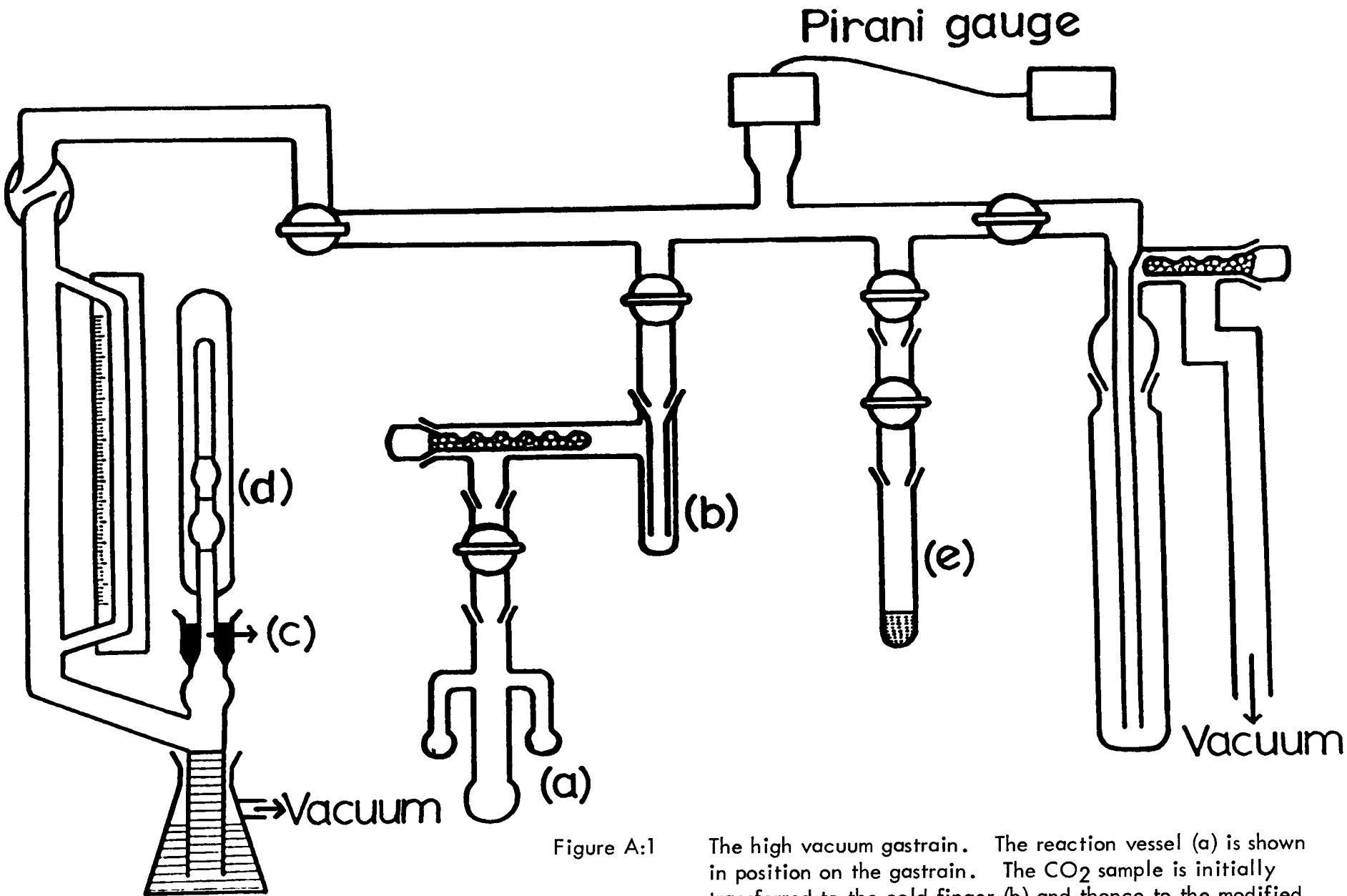


Figure A:1 The high vacuum gastrain. The reaction vessel (a) is shown in position on the gastrain. The CO_2 sample is initially transferred to the cold finger (b) and thence to the modified MacLeod chamber (d) by means of the liquid nitrogen cuff (c). After measuring its volume the sample of CO_2 is transferred to and trapped in the phenylethylamine–methanol mixture (e).

(xii) Raise mercury column to the reference mark on the modified MacLeod chamber and determine the scale reading indicated by the height of the mercury column in the manometer side-arm.

(xiii) Lower mercury column and transfer CO_2 sample to the tube containing 2 ml. of the phenylethylamine-methanol mixture frozen under liquid nitrogen.

(xiv) Remove tube and after thawing transfer solution to a counting vial containing 8 ml. of the P.P.O. - P.O.P.O.P. scintillation mixture.

D Calculation of Albumin Synthesis Rate.

I. Urea carbon specific activity measurements.

(i) Convert volumes of CO_2 into mg. C by means of previously constructed calibration graphs for known amounts of urea.

(ii) Calculate S.A. (expressed as counts per minute per mg. C) for each of urea samples.

(iii) Correct these S.A. measurements for the 2 mg. of stable urea added.

(iv) Plot corrected S.A. measurements on log-linear graph paper, (Figure A:2).

(v) By least mean squares method plot the best fitting line through these points.

(vi) Extrapolate line back to 'y' axis and determine urea S.A. at t_o.

(vii) From graph determine rate of fall of urea S.A. per hour i.e. fractional S.R. of urea per hour.

II. Albumin guanidine carbon S.A. measurement

(i) Calculate albumin S.A. as in I (i) and (ii) above.

(ii) Correct the S.A. measurement for the 100 mg. bovine albumin added.

(iii) Determine albumin S.A. t_o by multiplying the observed S.A. at t_6 by the factor derived from the disappearance of the iodinated albumin sample over the six hour experimental period.

Then / .....

Then,

$$\text{Fractional S.R. albumin (\% I.V.P. / hour)} = \frac{\text{fractional S.R. urea/hour} \times \text{albumin S.A. } t_o}{\text{urea S.A. } t_o}$$

and

$$\text{Absolute S.R. albumin (mg. / hour)} = \text{Fractional S.R. albumin} \times \text{intravascular albumin pool.}$$

WORKED EXAMPLE OF SYNTHESIS RATE CALCULATION

Hrs	Height of mercury column (cm.)	mg.C.	C.P.M.	S.A. (C.P.M./mg.C)		Corrected S.A. (C.P.M./mg.C)
3.0	20.2	0.24904	6927.9	27,818		245,633
3.5	20.5	0.25315	6740.5	26,627	Plasma urea = 67 mg/ 100 ml.	235,116
4.0	21.0	0.26000	6588.9	25,342		223,770
4.5	20.4	0.25178	6119.9	24,307	Stable urea added = 2.10 mg.	214,631
5.0	21.4	0.26548	5991.4	22,568		199,275
5.5	19.5	0.23945	5038.4	21.042	therefore correction factor is 8.83	185,801
6.0	20.3	0.25041	5054.6	20,185		178,234

From graph corrected urea S.A. t_o = 346,300

$$\text{Fractional S.R. urea (\%/hr)} = \frac{(\text{urea S.A. } t_o - \text{urea S.A. } t_1) \times 100}{\text{urea S.A. } t_o}$$

$$= \frac{(346{,}300 - 310{,}020) \times 100}{346{,}300}$$

$$= 10.48$$

Table / .....

Hrs.	Height of mercury column (cm.)	mg.C.	C.P.M.	S.A. (C.P.M./mg.C)		Corrected S.A. (C.P.M./mg.C)
6.0	14.5	0.17095	636.6	3,724	Plasma alb. = 1.73g/ 100 ml.	25,249
Samples in duplicate	13.9	0.16273	644.1	3,958	Stable alb. added = 100 mg therefore correction factor = 6.78	26,835

Mean corrected albumin S.A. t_6 = 26,042 c.p.m./mg.C.

The ^{125}I activity of an 0.02 ml. aliquot of plasma was observed to fall to 49.8% of its original value over the experimental period of 6 hours.

$$\text{Therefore corrected albumin S.A. } t_o = \frac{26,042 \times 100}{49.8}$$

$$= 52,293 \text{ c.p.m./mg.C}$$

$$\text{Fractional S.R. albumin (\% I.V.P./hr)} = \frac{52,293 \times 10.48}{346,300} = 1.58$$

Plasma albumin concentration = 1.73 g/100 ml

Plasma volume = 6.65 ml.

Intravascular albumin pool = 115.0 mg.

$$\text{Absolute S.R. albumin (mg/hour)} = \frac{1.58 \times 115.0}{100} = 1.8$$

Fractional and absolute synthesis rates were multiplied by 24 to give an average daily value.

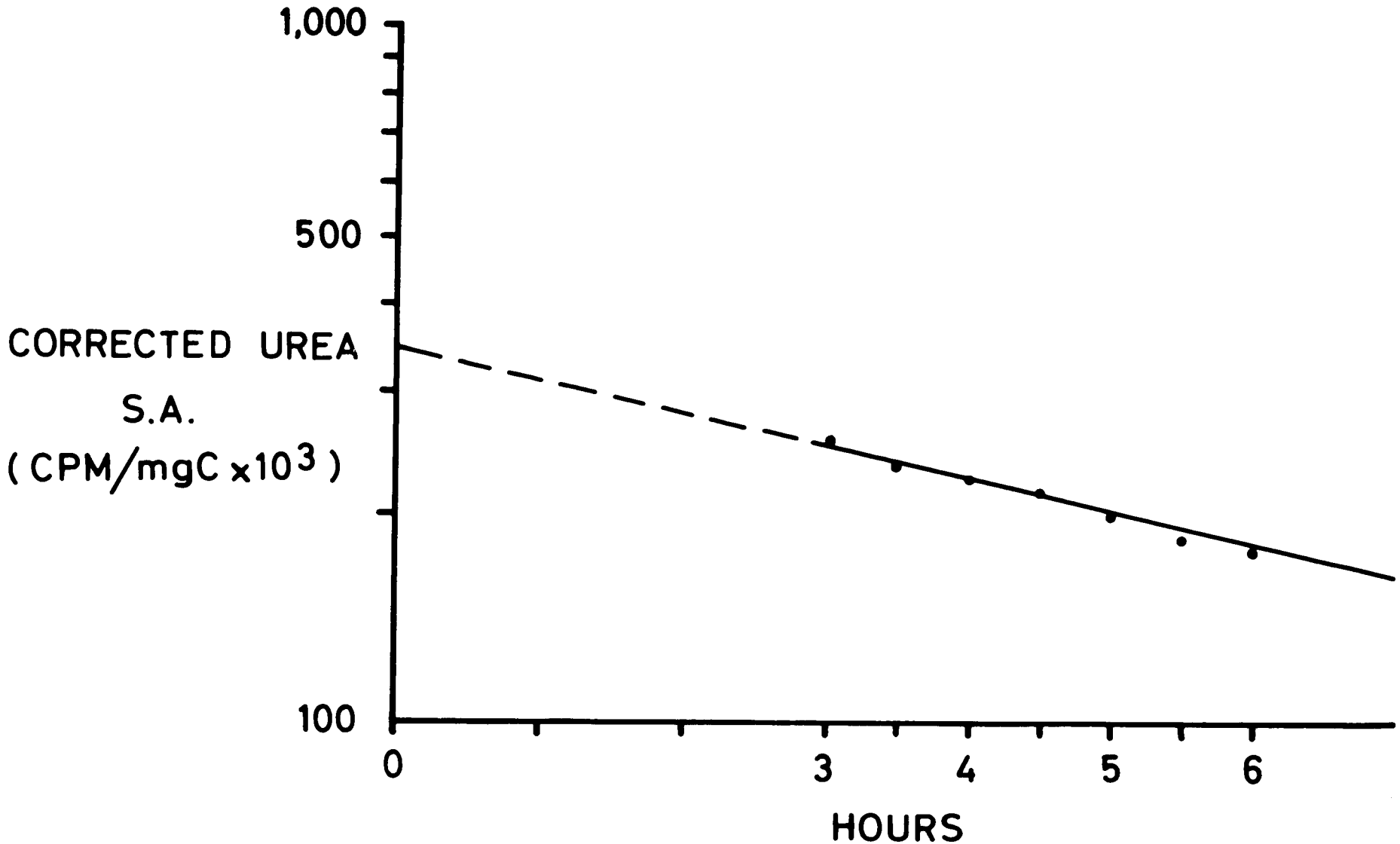


Figure A:2 Semi logarithmic plot of urea C plasma specific activities.

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